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**STUDIES ON THE HAEMOTOXICITY OF BUSULPHAN AND
CHLORAMPHENICOL IN THE B6C3F₁ MOUSE**

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**A thesis submitted to the University of London in partial fulfilment of the
requirements for the degree of Doctor of Philosophy**

November 2000

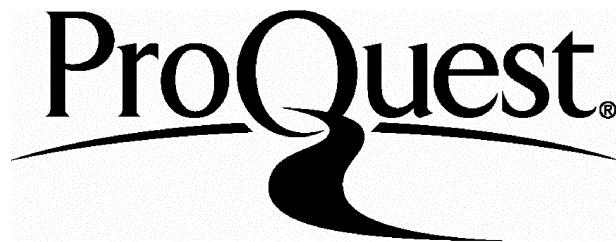
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ABSTRACT

Aplastic anaemia (AA) is a potentially fatal failure of haemopoiesis of unknown aetiology. In some cases, AA has been linked to drug administration, for example, chloramphenicol (CAP), phenylbutazone and gold salts. Recently, several drugs have been withdrawn following deaths from AA. There is at present no preclinical test, or animal model, predictive of drug-induced AA in man.

The objective of the present work was first, to investigate the haemotoxicity of busulphan (BU) and CAP (as succinate, CAPS) in the B6C3F₁ mouse, and second, to evaluate the toxicity of CAPS in BU-pretreated animals using standard haematological techniques.

The maximum tolerated single dose (MTD) of BU was 45 mg/kg; myelosuppression was maximal at 2 days (d) with recovery after 7 d. Macrocytic anaemia, thrombocytopenia and leucopenia, persisted at 14 d post dosing, and after four fortnightly BU doses of 40 mg/kg, at 2 and 6 weeks post dosing. CAPS given in the drinking water at 4.0 mg/mL caused reversible haemoconcentration with reticulocytopenia, but not myelosuppression.

BU was given at 40 mg/kg on d 1, 16, 30 and 43, followed by CAPS in the drinking water at 4.0 mg/mL from d 85 to 497. BU treatment alone resulted in deaths from aplasia up to d 168, thereafter deaths were due to lymphoma. Surviving BU-treated animals showed persistent thrombocytopenia and reductions in IgG⁺ lymphocytes, but no reticulocytopenia. No cumulative haemotoxic effect was seen in mice pretreated with BU and then given CAPS.

CAP kinetics were studied after administration in drinking water, by gavage and intravenous (iv) dosing. CAPS and CAP were quickly metabolised and cleared, and only very low concentrations reached the bone marrow. Gavage dosing at 3500 mg/kg/d for 5 d induced reversible myelosuppression, with recovery at 5-10 d after dosing.

In a second long-term experiment, BU was administered as before, but CAPS was gavaged at 3000 mg/kg/d, in 5-day pulses, in 21 d cycles. Deaths due to BU-induced aplasia and lymphoma were seen as before; no increased aplasia resulted from the administration of BU and CAPS.

It is concluded that administration of BU to B6C3F₁ mice induced aplasia in a proportion of animals. Giving high doses of CAPS by gavage caused reversible myelosuppression. However, administration of CAPS, following BU pretreatment, induced no increased haemotoxicity.

DEDICATION

For Kate

ACKNOWLEDGEMENTS

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CONTENTS

TITLE PAGE.....	1
ABSTRACT	2
DEDICATION.....	3
ACKNOWLEDGEMENTS.....	4
Index of Tables	10
Index of Figures.....	11
Abbreviations Used in This Thesis.....	13
CHAPTER 1: INTRODUCTION.....	16
1.1 Aplastic Anaemia in Man	16
1.1.1 The Condition	16
1.1.2 Incidence of Aplastic Anaemia.....	16
1.1.3 Diagnostic Criteria for Aplastic Anaemia.....	16
1.1.4 Laboratory Features of Aplastic Anaemia	19
1.1.4.1 Peripheral Blood	19
1.1.4.2 Bone Marrow	20
1.1.4.3 Cytokines	21
1.1.5 Disease Pathophysiology	22
1.1.5.1 Stem Cells	22
1.1.5.2 Bone Marrow Microenvironment	22
1.1.5.3 Direct Toxicity as a Causative Mechanism	23
1.1.5.4 Immune Mechanisms.....	23
1.1.6 Treatment of Aplastic Anaemia.....	25
1.1.6.1 Bone Marrow Transplantation	26
1.1.6.2 Immunosuppression	27
1.1.7 Outcome of Aplastic Anaemia.....	27
1.1.7.1 Late Outcome of Aplastic Anaemia.....	28
1.1.8 Acquired Aplastic Anaemia	29
1.1.8.1 Infections	30
1.1.8.2 Paroxysmal Nocturnal Haemoglobinuria.....	30
1.1.8.3 Pregnancy.....	31
1.1.8.4 Radiation	31
1.1.8.5 Drugs and Chemicals	32
1.1.8.5.1 Cancer Chemotherapeutic Agents.....	36
1.1.8.5.2 Benzene and Other Hydrocarbons	37
1.1.8.5.3 Chloramphenicol.....	38
1.1.8.5.4 Nonsteroidal Antiinflammatory Agents.....	40
1.1.8.5.5 Other Drugs.....	41
1.1.8.5.6 Drugs Recently Associated with Aplastic Anaemia	41
1.2 Aplastic Anaemia in Animals	43
1.2.1 Naturally-occurring Aplastic Anaemia	43
1.2.2 Experimentally-induced Aplastic Anaemia	44
1.2.2.1 Infective Agents and Immunosuppression	44
1.2.2.2 Radiation.....	45
1.2.2.3 Drugs and Chemicals	46
1.2.2.3.1 Benzene.....	46
1.2.2.3.2 Busulphan and Other Cytotoxic Agents	46
1.2.2.3.3 Chloramphenicol.....	47
1.2.2.3.4 Estrogens.....	47

1.2.2.3.5	Other Drugs and Chemicals	48
1.2.2.4	Genetic Models of Aplastic Anaemia	49
1.3	Chloramphenicol.....	51
1.3.1	Structure, Mode of Action, Therapeutic Indications	51
1.3.2	Chloramphenicol Toxicity	52
1.3.2.1	Type A Haemotoxicity.....	53
1.3.2.2	Type B Haemotoxicity	54
1.3.2.3	Toxicity in Man	56
1.3.2.4	Toxicity in Animals	57
1.4	Busulphan	59
1.4.1	Structure, Mode of Action, Therapeutic Indications	59
1.4.2	Toxicity in Man	60
1.4.3	Toxicity in Animals	61
1.4.4	Haemotoxicity.....	62
1.5	The Investigations of AA Morley and the Development of a Mouse Model of Chronic Hypoplastic Marrow Failure.....	63
1.6	Objectives of the Present Work	67
 CHAPTER 2: MATERIALS AND METHODS		
2.1	Animals	68
2.2	Dosing	68
2.2.1	Busulphan	68
2.2.2	Chloramphenicol Succinate	68
2.3	Haematological Measurements	69
2.3.1	Blood Samples	69
2.3.2	Peripheral Blood: Full Blood Count, Differential Leucocyte Count and Reticulocyte Count	69
2.3.3	Bone Marrow	71
2.3.4	Spleen.....	71
2.4	Flow Cytometry	71
2.4.1	Lymphocyte Subset Analysis.....	71
2.4.1.1	Method 1	72
2.4.1.2	Method 2	72
2.4.2	Bone Marrow Stem Cell Analysis	73
2.5	Bone Marrow Progenitor Cell Assays	73
2.6	Histopathology.....	74
2.7	Chloramphenicol and Chloramphenicol Succinate Determination.....	74
2.7.1	Method 1	74
2.7.2	Method 2	75
2.8	Osmolality.....	75
2.9	<i>In vitro</i> Haemolysis Tesing.....	75
2.10	Statistical Analysis.....	75
 CHAPTER 3: DOSE RANGING STUDIES WITH BUSULPHAN: CHARACTERISATION OF SHORT-TERM MYELOTOXICITY		
3.1	Introduction.....	77
3.2	Materials and Methods.....	79
3.2.1	Experiment 1: Busulphan Dose Ranging: Single Dose Study with Sampling at 14 Days	80

3.2.2	Experiment 2: Busulphan Dose Ranging: Repeat Dose Study with Sampling at 14 and 42 Days.....	80
3.2.3	Experiment 3: Acute Toxicity of Busulphan: 10-day Study with Sequential Sampling Following a Single Dose	80
3.3	Results.....	81
3.3.1	Experiment 1: Busulphan Dose Ranging: Single Dose Study with Sampling at 14 Days	81
3.3.1.1	Clinical Observations.....	81
3.3.1.2	Haematological Measurements.....	81
3.3.2	Experiment 2: Busulphan Dose Ranging: Repeat Dose Study with Sampling at 14 and 42 Days.....	85
3.3.2.1	Clinical Signs.....	85
3.3.2.2	Haematology	85
3.3.3	Experiment 3: Acute Toxicity of Busulphan: 10-day Study with Sequential Sampling Following a Single Dose	89
3.3.3.1	Clinical Signs.....	89
3.3.3.2	Haematology	89
3.4	Discussion.....	97

CHAPTER 4: CHLORAMPHENICOL SUCCINATE ADMINISTRATION IN THE DRINKING WATER: DOSE RANGING STUDIES AND HAEMATOLOGICAL EFFECTS

4.1	Introduction.....	103
4.2	Materials and Methods.....	104
4.2.1	Experiment 4: Determination of the Maximum Tolerated Dose of Chloramphenicol Succinate in the Drinking Water: A Seven-day Study....	105
4.2.2	Experiment 5: Haematological Effects of Chloramphenicol Succinate in the Drinking Water: a 30-day Study	105
4.3	Results.....	106
4.3.1	Experiment 4: Determination of the Maximum Tolerated Dose of Chloramphenicol Succinate in the Drinking Water: A Seven-day Study....	106
4.3.1.1	Clinical Observations, Water Consumption and Body Weights.....	106
4.3.1.2	Haematology	108
4.3.2	Experiment 5: Haematological Effects of Chloramphenicol Succinate in the Drinking Water: a 30-day Study	109
4.3.2.1	Clinical Observations, Water Consumption and Body Weights.....	109
4.3.2.2	Haematology	111
4.4	Discussion.....	113

CHAPTER 5: EVALUATION OF BUSULPHAN AND CHLORAMPHENICOL SUCCINATE IN THE INDUCTION OF APLASTIC ANAEMIA IN THE B6C3F₁ MOUSE

5.1	Introduction.....	117
5.2	Materials and Methods.....	118
5.2.1	Experiment 6: The Administration of Busulphan and Chloramphenicol Succinate to B6C3F ₁ Mice: a 497-day Study	119
5.3	Results.....	120
5.3.1	Mortality, Clinical Signs and Haematology of Intercurrent Death Animals	120
5.3.1.2	Influence of Chloramphenicol Succinate on Mortality.....	123
5.3.1.3	Influence of Busulphan on Mortality	130

5.3.2	Haematology	132
5.3.2.1	Bone Marrow and Spleen.....	135
5.3.2.2	Erythrocytes and Platelets.....	138
5.3.2.3	Leucocytes	141
5.3.3	Histopathological Findings	145
5.3.3.1	Three Animals with Marrow Aplasia, Sampled on Days 107, 129 and 168	145
5.3.3.2	Four Animals with Lymphoma, Sampled on Days 175, 184, 191 and 217..	147
5.3.3.3	Scheduled Autopsy at Day 85.....	149
5.3.3.4	Scheduled Autopsy at Day 384.....	149
5.3.4	Chloramphenicol and Chloramphenicol Succinate Analysis.....	152
5.4	Discussion.....	155
CHAPTER 6: INVESTIGATIONS INTO CHLORAMPHENICOL KINETICS		165
6.1	Introduction.....	165
6.2	Materials and Methods.....	166
6.2.1	Experiment 7: Periodicity of Plasma Levels of Chloramphenicol and Chloramphenicol Succinate when Chloramphenicol Succinate was Administered in the Drinking Water.	166
6.2.2	Experiment 8: Kinetics of a Single Intravenous Dose of Chloramphenicol Succinate	167
6.2.3	Experiment 9: Kinetics of Chloramphenicol Succinate Administered by Gavage	167
6.3	Results.....	168
6.3.1	Experiment 7: Periodicity of Serum Levels of Chloramphenicol and Chloramphenicol Succinate when Chloramphenicol Succinate was Administered in the Drinking Water.....	168
6.3.1.1	Clinical Observations and Body Weight.....	168
6.3.1.2	Water Consumption	169
6.3.1.3	Serum Concentrations of Chloramphenicol Succinate and Chloramphenicol.....	170
6.3.1.3.1	Chloramphenicol Succinate	170
6.3.1.3.2	Chloramphenicol.....	170
6.3.1.4	Haematology	173
6.3.2	Experiment 8: Kinetics of a Single Intravenous Dose of Chloramphenicol Succinate	176
6.3.2.1	Clinical Observations.....	176
6.3.2.2	Haematology	176
6.3.2.3	Osmolality and <i>In vitro</i> Haemolysis with Chloramphenicol Succinate Solutions	179
6.3.2.4	Serum, Liver and Bone Marrow Chloramphenicol Succinate and Chloramphenicol Concentrations.....	182
6.3.3	Experiment 9: Kinetics of Chloramphenicol Succinate Administered by Gavage	185
6.3.3.1	Clinical Observations.....	185
6.3.3.2	Serum and Bone Marrow Chloramphenicol and Chloramphenicol Succinate Concentrations.....	185
6.4	Discussion.....	187

CHAPTER 7: CHARACTERISATION OF THE MYELOTOXICITY INDUCED BY THE ORAL ADMINISTRATION OF HIGH DOSE LEVELS OF CHLORAMPHENICOL SUCCINATE	193
7.1 Introduction.....	193
7.2 Materials and Methods.....	194
7.2.1 Experiment 10: 25-day Gavage Study with High Dose Chloramphenicol Succinate	194
7.3 Results.....	195
7.3.1 Clinical Observations.....	195
7.3.2 Haematology: Erythroid Series.....	195
7.3.3 Haematology: Leucocytes.....	200
7.4 Discussion.....	204
CHAPTER 8: THE HAEMOTOXICITY OF BUSULPHAN AND HIGH DOSE CHLORAMPHENICOL SUCCINATE IN THE B6C3F₁ MOUSE.....	208
8.1 Introduction.....	209
8.2 Materials and Methods.....	209
8.2.1 Experiment 11: 252-day Study with Busulphan Administration and Intermittent High Dose Chloramphenicol Succinate	209
8.3 Results.....	210
8.3.1 Survival	210
8.3.1.1 Analysis of the Influence of Chloramphenicol Succinate Administration on Intercurrent Deaths.....	212
8.3.1.2 Intercurrent Deaths to day 156: Haematology	213
8.3.1.3 Intercurrent Deaths after day 156	216
8.3.2 Scheduled Bleeds: Haematology	216
8.3.2.1 Effects of Chloramphenicol Succinate (Group 2).....	217
8.3.2.2 Effects of Busulphan (Group 3).....	220
8.3.2.3 Combined Effects of Busulphan and Chloramphenicol Succinate (Group 4).....	221
8.4 Discussion.....	222
CHAPTER 9: FINAL DISCUSSION.....	229
9.1 Summary of Findings in the Present Work.....	229
9.1.1 Characterisation of BU and CAPS Haemotoxicity in the B6C3F ₁ Mouse ...	229
9.1.2 Considerations Related to the Validation of a Model of Chloramphenicol-induced Aplastic Anaemia in the B6C3F ₁ Mouse	230
9.2 Potential Future Investigations: Busulphan- and CAP-induced Haemopoietic Injury in the Mouse: Models for Human Disease	232
9.3 Future Strategies for the Investigation of Idiosyncratic Drug-induced AA in Man	236
9.3.1 Metabolic Predisposition to Drug-induced Aplastic Anaemia	236
REFERENCES.....	240
APPENDIX: PUBLISHED WORK RELEVANT TO THIS THESIS	305

Index of Tables

Table 1.1: Criteria for aplastic anaemia	18
Table 1.2: Criteria for severe aplastic anaemia	18
Table 1.3: Summary of laboratory features of aplastic anaemia	19
Table 1.4: Epidemiological classification of aplastic anaemia.....	29
Table 1.5: Classification of drugs and chemicals associated with aplastic anaemia	33
Table 1.6: Drug and chemical association with aplastic anaemia: clinical series.....	34
Table 1.7: Aplastic anaemia: use of drugs 29 - 180 days before admission.....	35
Table 1.9: Epidemiological studies of chloramphenicol and aplastic anaemia	39
Table 4.1: Daily consumption of CAPS in mice receiving CAPS in drinking water for 7 d.....	105
Table 5.1: Design of Experiment 6.	119
Table 5.2: Individual haematological values of ICD mice treated with BU on d 1, 16, 30 and 43.	121
Table 5.2 (continued)	122
Table 5.3: The influence of CAPS administration on the occurrence of ICDs due to lymphoma.....	129
Table 5.4: Statistical analysis of haematological parameters in Experiment 6.....	133
Table 5.5: Combined platelet counts from mice sampled on d 140	133
Table 5.6: Femoral nucleated cells in mice treated with CAPS alone and BU + CAPS.	136
Table 5.7: Splenic nucleated cell counts in mice treated with with CAPS alone and BU + CAPS.	136
Table 5.8: Estimated consumption of CAPS in group 7 mice.....	153
Table 5.9: Serum CAP levels in samples from individual mice killed on d 489.	153
Table 5.10: CAPS concentrations in stock CAPS solution and dosing solution	153
Table 6.1: Serum CAPS concentrations in mice receiving CAPS in the drinking water for 14 d.....	171
Table 6.2: Serum CAP concentrations in mice receiving CAPS in drinking water for 14 d.....	172
Table 6.3: Haematological measurements in mice 15 minutes after iv administration of CAPS.....	177
Table 6.4: Concentrations of CAPS in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS.....	183
Table 6.5: Concentrations of CAP in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS.....	184
Table 6.6: Serum CAP concentrations in mice following a single oral CAPS.....	186
Table 7.1: Erythroid parameters following CAPS gavage at 2500 and 3500 mg/kg.....	196
Table 7.2: Leucocyte parameters following CAPS gavage at 2500 and 3500 mg/kg.	202
Table 8.1: Design of Experiment 11.	210
Table 8.2: Incidence of intercurrent deaths in BU-treated mice.....	212
Table 8.3: Peripheral cell counts, femoral and spleen nucleated counts and spleen weight in ICD mice ...	214
Table 8.4: Peripheral cell counts, femoral and spleen nucleated counts and spleen weight in three ICD mice dying on d 189, 192 and 239.	215
Table 8.5: Effect of BU and CAPS on erythrocyte measurements and platelet counts, leucocyte counts, femoral bone marrow counts, stem cell counts, and colony-forming unit assays, spleen counts and spleen weights.....	218

Index of Figures

Figure 1.1: Chemical structure of CAP	51
Figure 1.2: Chemical structure of CAP, TAP and nitroso-CAP	55
Figure 1.3: Chemical structure of BU	59
Figure 3.1: Erythrocyte count, reticulocytes and macrocytic cells at 14 d after a single BU dose.....	82
Figure 3.2: Platelet counts at 14 d after a single BU dose.....	83
Figure 3.3: Total leucocyte and lymphocyte counts at 14 d after a single BU dose.....	83
Figure 3.4: Neutrophil counts at 14 d after a single BU dose.....	84
Figure 3.5: Leucocyte counts, other than neutrophils and lymphocytes, at 14 d after a single BU dose	84
Figure 3.6: Erythrocyte counts, reticulocyte and macrocyte percentages at 14 and 42 d after four fortnightly BU doses.	86
Figure 3.7: Platelet counts at 14 and 42 d after four fortnightly BU doses.	86
Figure 3.8: Lymphocyte counts at 14 and 42 d after four fortnightly BU doses.	87
Figure 3.9: Mean and sd of neutrophil counts at 14 and 42 d after four fortnightly BU doses.	88
Figure 3.10: Erythrocyte count, reticulocytes and macrocytic cells following a single BU dose of 45 mg/kg.....	90
Figure 3.11: Femoral marrow erythroid precursors and reticulocyte count following a single BU dose of 45 mg/kg.	90
Figure 3.12: Femoral marrow and splenic nucleated cell counts following a single BU dose of 45 mg/kg..	91
Figure 3.13: Femoral bone marrow smears at timepoints following a single dose of 45 mg BU/kg.	92
Figure 3.14: Summary of erythroid series changes following a single BU dose of 45 mg/kg	94
Figure 3.15: Leucocyte and bone marrow myeloid cell count following a single BU dose of 45 mg/kg.....	96
Figure 3.16: Neutrophil count and MPXI following a single BU dose of 45 mg/kg.....	96
Figure 4.1: Water consumption in mice receiving CAPS in the drinking water for 7 d.....	106
Figure 4.2: Body weights in mice receiving CAPS in drinking water at for 7 d.	107
Figure 4.3: Erythrocyte and reticulocyte counts in mice receiving CAPS in drinking water for 7 d.	108
Figure 4.4: Daily water consumption per mouse receiving water alone or CAPS for 30 d.....	110
Figure 4.5: Body weights of mice receiving tap water or CAPS in the drinking water for 30 d.....	110
Figure 4.6: Erythrocyte counts in mice receiving tap water or CAPS in the drinking water for 30d.....	112
Figure 4.7: Reticulocyte counts in mice receiving tap water or CAPS in the drinking water for 30 d.....	112
Figure 4.8: Erythrocyte count, reticulocyte count and body weights in mice receiving 4.0 mg CAPS/mL in drinking water for up to 30 d.....	113
Figure 5.1: Peripheral blood and bone marrow morphology in BU-treated aplastic ICD mice.	125
Figure 5.2 (a): Bayer H*1 cytograms.....	126
Figure 5.2 (b): Bayer H*1 cytograms.....	127
Figure 5.3: Morphological features in peripheral blood, bone marrow and spleen in lymphoma bearing ICD mice	128
Figure 5.4: Survival of mice dosed with BU.....	130
Figure 5.5: Fur greying in BU-treated mice.	131
Figure 5.6: Representative plots of haemoglobin and peripheral cell counts in mice treated with BU....	134
Figure 5.7: Haemoglobin, reticulocyte percentage and platelet counts in mice treated with four BU doses.....	140
Figure 5.8: Total leucocyte, neutrophil and lymphocyte counts in mice treated with four BU doses.	143
Figure 5.9: Lymphocyte subset counts on d 485 in mice given four BU doses	144
Figure 5.10: Lymphoreticular abnormalities in aplastic ICD mice in Experiment 6.....	146
Figure 5.11: Tissue infiltration in lymphoma bearing ICD mice in Experiment 6.....	148
Figure 5.12: Histological abnormalities in BU-treated mice at d 384 of Experiment 6.	150
Figure 6.1: Water consumption in mice receiving CAPS in the drinking water.	169
Figure 6.2: Serum CAPS concentrations in mice receiving CAPS in drinking water for 14 d and sampled at four timepoints over a 16 h period.....	171
Figure 6.3: Serum CAP concentrations in mice receiving CAPS in the drinking water for 14 d and sampled at four timepoints over a 16 h period.....	172
Figure 6.4: Reticulocyte counts in mice receiving CAPS in drinking water for 14 d and sampled at four timepoints over a 16 h period.....	174
Figure 6.5: Spleen nucleated cell counts, total leucocyte, lymphocyte and neutrophil counts in control mice sampled at four timepoints over a 16 h period.	174

Figure 6.6: Platelet counts in mice receiving CAPS in drinking water for 14 d sampled at four timepoints over a 16 h period	175
Figure 6.7: Effects of iv dosing with CAPS.	178
Figure 6.8: <i>In vitro</i> haemolysis of human and mouse blood with aqueous and saline CAPS solutions	180
Figure 6.9: <i>In vitro</i> haemolysis of human and mouse blood with aqueous and saline CAPS solutions.	180
Figure 6.10: Osmolality of saline and aqueous solutions of CAPS.....	181
Figure 6.11: Concentration of CAPS in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS.....	183
Figure 6.12: Concentration of CAP in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS	184
Figure 6.13: Serum CAP concentrations in mice following a single oral dose of CAPS,	186
Figure 7.1 Vacuolation in bone marrow smears of mice receiving five daily doses of 2500 mg CAPS/kg by gavage.....	197
Figure 7.2: Comparison of splenic erythropoietic activity with HFR reticulocyte count.....	198
Figure 7.3: Response of erythroid elements and platelets in mice receiving 5 doses of CAPS at 2500 mg/kg/d.	199
Figure 7.4: Response of erythroid elements and platelets in mice receiving 5 doses of CAPS at 3500 mg/kg/d.	199
Figure 7.5: Response of myeloid elements in mice receiving 5 daily doses of CAPS at 2500 mg/kg/d.	203
Figure 7.6: Response of myeloid elements in mice receiving 5 doses of CAPS at 3500 mg/kg/d	203
Figure 8.1: Survival in BU-treated animals.....	211
Figure 8.2: Erythrocytes, reticulocytes, macrocytes and microcytes in mice on d 252 following four fortnightly BU doses and intermittent CAPS dosing.....	217

Abbreviations Used in This Thesis

AA	Aplastic anaemia
AIDS	Acquired immunodeficiency syndrome
ALS/G	Antilymphocyte serum/globulin
AML	Acute myeloblastic leukaemia
ATG	Antithymocyte globulin
AZT	Azidothymidine
BALT	Bronchial associated lymphoid tissue
Baso	Basophil(s)
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BFU-E	Burst-forming unit, erythroid
BM	Bone marrow
BMH	Bone marrow hypoplasia
BMT	Bone marrow transplant(ation)
BU	Busulphan
CAP(S)	Chloramphenicol (succinate)
CD	Cluster of differentiation
CFC	Colony-forming cell
CFU	Colony-forming unit
CFU-C	Colony-forming unit, culture (CFU-GM)
CFU-E	Colony-forming unit, erythroid
CFU-F	Colony-forming unit, fibroblast
CFU-GM	Colony-forming unit, granulocyte/macrophage
CFU-Meg	Colony-forming unit, megakaryocytic
CFU-S	Colony-forming unit, spleen
CHMF	Chronic hypoplastic marrow failure
CML	Chronic myelogenous leukaemia
CN	Cyclical neutropenia
CY	Cyclophosphamide
CYP	Cytochrome P450
d	day(s)
EB	3,4-epoxybutene
EDTA	Ethylene diaminotetraacetic acid
EMA	European Agency for the Evaluation of Medicinal Products
EORTC	European Organisation for Research on Treatment of Cancer
Eos	Eosinophil(s)
EPO	Erythropoietin
FITC	Fluorescein isothiocyanate
FL	Flt3 ligand
G6PDH	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
Gran	Granulocyte(s)
GVHD	Graft-versus-host disease
Hb	Haemoglobin
HCT	Haematocrit
HDW	Haemoglobin (cellular concentration) distribution width
H&E	Haematoxylin and eosin

HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HFR	High fluorescence ratio reticulocytes (also MFR, LFR: Mid-, Low FR)
HLA	Human leucocyte antigen
HPLC	High performance liquid chromatography
HSC	Haemopoietic stem cell
HU	Hydroxyurea
Hypo	Hypochromic erythrocyte(s)
IAAAS	International Agranulocytosis and Aplastic Anemia Study
IARC	International Agency for Research in Cancer
ICD	Intercurrent death
IFN	Interferon
IL	Interleukin
ip	intraperitoneal(ly)
iv	intravenous(ly)
LD	Lethal dose (as in LD ₅₀)
LTC-IC	Long-term culture initiating cell
LUC	Large unstained cell(s)
Lymph	Lymphocyte(s)
Macro	Macrocytic erythrocyte(s)
MCH	Mean cell (erythrocyte) haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MDS	Myelodysplastic syndrome
MGG	May-Grünwald-Giemsa
min	minute(s)
MMC	Mitomycin C
MN	Mononuclear (leucocytes)
Mono	Monocytes(s)
MPO	Myeloperoxidase
MPXI	(Neutrophil) Myeloperoxidase index
MTD	Maximum tolerated dose
MTSD	Maximum tolerated single dose
NADH	Reduced nicotinamide-adenine-dinucleotide
NAP	Neutrophil alkaline phosphatase
Neut	Neutrophil(s)
NK	Natural killer
ns	not (statistically) significant
NSAID	Non-steroidal antiinflammatory drug
PBSC	Peripheral blood stem cell
PBZ	Phenylbutazone
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PLT	Platelets
PMN	Polymorphonuclear (leucocytes)
PNH	Paroxysmal nocturnal haemoglobinuria
PRCA	Pure red cell aplasia
PRV	Polycythaemia rubra vera
PSC	Pluripotent stem cell
RBC	Red blood cell (erythrocyte) count
RDW	Red cell (volume) distribution width

rm	recombinant murine
RPMI	Roswell Park Memorial Institute
Retic	Reticulocyte
SAA	Severe aplastic anaemia
sc	Subcutaneous(ly)
SCF	Stem cell factor
sd	standard deviation
TGF	Transforming growth factor
TNC	Total nucleated cell count
TNF	Tumour necrosis factor
TPO	Thrombopoietin
USNTP	United States National Toxicology Program
VSAA	Very severe aplastic anaemia
WBC	White blood cell (leucocyte) count

CHAPTER 1: INTRODUCTION

1.1 Aplastic Anaemia in Man

1.1.1 The Condition

Aplastic anaemia (AA) is a failure of haemopoiesis in all three bone marrow derived lineages - myeloid, erythroid and platelets. Ehrlich (Ehrlich, 1888) erroneously described AA as a variant of pernicious (megaloblastic) anaemia. However, Vaquez and Aubertin, noting the fatty appearance of the marrow, described the lack of haemopoiesis as "aplastic" (Vaquez and Aubertin, 1904). The term "aplastic anaemia" was later applied by Chauffard, although "aplastic pancytopenia" would be a more accurate description (Camitta *et al.*, 1982a).

1.1.2 Incidence of Aplastic Anaemia

AA is now recognised as having a prevalence about 2 per million of population per annum in European countries (IAAAS, 1986, 1987; Mary *et al.*, 1990; Kaufman *et al.*, 1991, 1996). It is equally frequent in males and females, the median age of onset being between 20 and 25 years. However, a second peak of incidence is seen in patients aged 60 years and above (Young, 1995). AA is recognised as being more prevalent in the Orient compared to the West (Young *et al.*, 1986) where risk factors such as socio-economic status, grain farming and exposure to solvents, rather than any genetic differences, lead to a rate of 3.9 - 4.1 per million in Thailand (Issaragrisil *et al.*, 1996).

1.1.3 Diagnostic Criteria for Aplastic Anaemia

Diagnosis of AA is based on the finding of bone marrow hypoplasia from the assessment of a trephine biopsy (Gordon-Smith 1989). A smear made from marrow aspiration may

mislead, in that cellularity in AA often varies considerably, with isolated foci of normal or even increased cellularity interspersed amidst areas of hypocellularity (Lewis, 1972).

Criteria for the definite establishment of a diagnosis of AA are important for two reasons.

Firstly, the disease itself must be differentiated from other entities, such as

myelodysplastic syndromes (MDS) or fulminant leukaemia (Guinan, 1997). Secondly, the degree of aplasia at presentation is a good indicator for patient outcome, selection of treatment, response to therapy and survival (Lewis, 1965; Lynch *et al.*, 1975) and allows better comparison of treatment modalities.

The stringency of these criteria have brought about a refinement of diagnosis, and called into question earlier surveys which are thought to have overestimated the prevalence of AA, which may have included cases of bone marrow failure other than AA. The use of strict diagnostic criteria excluded 46.3 % of 315 cases originally notified as AA in a recent survey (IAAAS, 1987). Patients can be divided into groups of non-severe AA, in which recovery is approximately 50 % (Table 1.1), and severe aplastic anaemia (SAA; Table 1.2), whose chances of recovery are about 10 % (Gordon-Smith, 1989). In addition, a category of very severe aplastic anaemia (VSAA) has been assigned to patients presenting with infection and a neutrophil count of less than $0.2 \times 10^9/L$ (Bacigalupo *et al.*, 1986). Whilst agreement between authors is close, there still exist minor diagnostic discrepancies (Benichou and Solal Celigny, 1991; Mary *et al.*, 1996; Young and Maciejewski, 1997).

Table 1.1: Criteria for aplastic anaemia*

At least two of the following peripheral blood features should be present:
Haemoglobin ≤ 10 g/dL or haematocrit ≤ 0.30 together with reticulocyte count $\leq 30 \times 10^9/L$;
Leucocyte count $\leq 3.5 \times 10^9/L$ or granulocytes $\leq 1.5 \times 10^9/L$;
Platelet count $\leq 50 \times 10^9/L$

For confirmation of the diagnosis, an adequate bone marrow biopsy should show a decrease in cellularity with the absence or depletion of all cells, or normal cellularity due to focal erythroid hyperplasia, with depletion of granulopoietic cells and megakaryocytes and the absence of significant fibrosis or neoplastic infiltration.

*IAAAS, 1987; Kaufman *et al.*, 1996

Table 1.2: Criteria for severe aplastic anaemia*

Peripheral blood: pancytopenia with at least two of the following:
Neutrophils $<0.5 \times 10^9/L$
Platelets $<20 \times 10^9/L$
Reticulocytes $<1\%$ (corrected for haematocrit**)
Bone marrow: markedly hypoplastic: $<25\%$ normal; or moderately hypoplastic: 25-50 % of normal cellularity with less than 30 % remaining cells haemopoietic.

*Camitta *et al.* (1976, 1982b)

**Corrected reticulocyte percentage = actual percentage \times (actual haematocrit / normal haematocrit for age)

1.1.4 Laboratory Features of Aplastic Anaemia

1.1.4.1 Peripheral Blood

The morphological features of peripheral blood in AA are nonspecific (Table 1.3). The anaemia may be macrocytic, particularly if there is still some residual haemopoietic function, and red cell lifespan is often reduced (Lewis, 1962).

Table 1.3: Summary of laboratory features of aplastic anaemia

	Cells		Cytokines	
Peripheral blood	Granulocytes	decreased	EPO	increased
	NAP	increased	TPO	increased
	Platelets	decreased	G-CSF	increased
	Reticulocytes	decreased	SCF	variable
	MCV	increased	FL	increased
	B lymphocytes	decreased	IFN γ	increased*
	T lymphocytes	(increased)	TNF α	increased*
Bone marrow	Cellularity	decreased	G-CSF	normal
	CD34 $^{+}$	decreased	GM-CSF	normal
	CD33 $^{+}$ CD34 $^{+}$	decreased	IL-6	normal
	LTC-IC	decreased	SCF	variable
	CFU-GM	decreased		
	CFU-mix	decreased		
	BFU-E	decreased		
	CFU-F	normal		

Abbreviations: NAP, neutrophil alkaline phosphatase; MCV, mean cell volume; EPO, erythropoietin; TPO, thrombopoietin; G-CSF, granulocyte colony-stimulating factor; SCF, stem cell factor; FL, Flt-3 ligand; IFN γ , interferon- γ ; IL-, interleukin; TGF β , transforming growth factor- β ; CD, cluster of differentiation; LTC-IC, long-term culture-initiating cell; CFU-GM, colony-forming unit, granulocyte/macrophage; CFU-mix, CFU, mixed lineage; BFU-E, burst-forming unit, erythroid; CFU-F, CFU, fibroblast; GM-CSF, granulocyte/macrophage CSF.

*by PHA stimulation.

Some degree of anisocytosis may be present, and neutrophils can show an increase in size and number of granules, giving the appearance of toxic granulation. The neutrophil alkaline phosphatase (NAP) score is raised (Gordon-Smith, 1989).

The proportion of B lymphocytes is often reduced in the peripheral blood of AA patients (Morley *et al.*, 1974b) and there may be increased numbers of morphologically activated lymphocytes, which may reflect a slight proportional increase in the number of HLA-DR+ (activated) CD8⁺ cells (Viale *et al.*, 1991), although this increase, and the increased numbers of CD8⁻ CD56⁺ cells (natural killer, NK cells) may only be detectable in the bone marrow of some patients (Zoumbos *et al.*, 1985; Maciejewski *et al.*, 1994).

1.1.4.2 **Bone Marrow**

If sufficient bone marrow (BM) is available for morphological study, dyserythropoietic features, such as cytoplasmic vacuolation, and normoblast pyknosis, nuclear fragmentation and multinucleation may be observed (Lewis, 1972). Numbers of bone marrow CD34⁺ cells (Marsh *et al.*, 1991), CD33⁻CD34⁺ cells (Scopes *et al.*, 1994) and a surrogate for early stem cells, long-term culture-initiating cells (LTC-IC; Maciejewski *et al.*, 1996), are markedly depleted in AA (Table 1.3). When CD34⁺ cells from AA patients are cultured with normal stromal cells in long-term marrow culture systems (Dexter and Lajtha, 1974; Dexter *et al.*, 1977), no colonies develop, whereas AA stromal cells are usually able to support haemopoiesis by CD34⁺ cells from normal donors (Marsh *et al.*, 1990). Bone marrow CFU-GM (colony-forming units, granulocyte/macrophage), CFU-mix (mixed lineage CFU) and BFU-E (burst-forming units, erythroid) in long-term culture are significantly lower in untreated AA patients. Antilymphocyte globulin (ALG) treatment increases the numbers of CFU-GM, which are still abnormally low, but does not affect CFU-mix or BFU-E (Marsh *et al.*, 1990). Normal numbers of fibroblast colonies (CFU-F) are found in long-term cultures of untreated AA BM (Marsh *et al.*, 1990).

1.1.4.3 Cytokines

Elevated levels of erythropoietin (EPO), disproportionately high for the degree of anaemia (Das *et al.*, 1992), are seen in AA (Table 1.3). Thrombopoietin levels are markedly elevated in responding AA patients prior to immunosuppressive therapy (Emmons *et al.*, 1996). Granulocyte colony-stimulating factor (G-CSF), which promotes the differentiation and growth of granulocytes, is found at elevated concentrations in the serum, increasing with the degree of neutropenia (Watari *et al.*, 1989). However, G-CSF, GM-CSF and interleukin 6 (IL-6) are present in normal concentrations in unstimulated, cultured, marrow stromal cells of AA patients but are at normal or elevated levels after stimulation with IL-1 (Kojima *et al.*, 1992). Similar amounts of GM-CSF are produced by phytohaemagglutinin (PHA) stimulated peripheral lymphocytes in AA patients and normal controls (Nimer *et al.*, 1991). On the other hand, in a minority of patients, reduced stromal levels of GM-CSF, IL-3 and G-CSF are found (Migliaccio *et al.*, 1992; Tani *et al.*, 1993). Levels of IL-1 in stimulated peripheral blood mononuclear cells and monocytes are generally reduced, and in patients with profound neutropenia, markedly so (Nakao *et al.*, 1989). However, this may reflect a more general maturation defect of monocytes (Young and Maciejewski, 1997). Stem cell factor (SCF) levels in SAA have been found to be generally low to normal, and below the lower limit of the normal range in about 20 % of patients. Normal, or even high, levels of SCF were associated with early remission, whilst low levels were associated with greater mortality. SCF levels in peripheral blood were predictive of the bone marrow SCF concentration (Wodnar-Filipowicz *et al.*, 1993). More recently, Kojima *et al.*, (1997) found no difference in plasma SCF levels between AA patients and controls. Flt-3 ligand (FL), which acts at a very early stage of haemopoiesis, is similarly seen at very high levels in the serum of AA patients (Lyman *et al.*, 1995). Transforming growth factor- β (TGF β), a suppressor of

haemopoiesis which acts antagonistically to FL, is significantly reduced in the serum and cultured stroma of AA patients (Rizzo *et al.*, 1999). Levels of interferon- γ (IFN γ) and tumour necrosis factor (TNF) from phytohaemmagglutinin-induced peripheral blood mononuclear cells are greatly increased in SAA (Hinterberger *et al.*, 1988).

1.1.5 Disease Pathophysiology

1.1.5.1 Stem Cells

The absolute number of haemopoietic stem cells (HSC) in the bone marrow of AA patients is considerably reduced, as is their capability to respond to a variety of proliferative stimuli (Boggs and Boggs, 1976; Marsh *et al.*, 1990, 1991; Scopes *et al.*, 1994; Maciejewski *et al.*, 1996). Increased apoptosis is seen in BM CD34 $^{+}$ cells (Philpott *et al.*, 1995). However, the mechanism underlying these defects remains unclear.

1.1.5.2 Bone Marrow Microenvironment

Because, in some patients, pockets of active haemopoietic marrow persisted, but failed to repopulate the surrounding aplastic sites, it was argued that “soil” (the bone marrow microenvironment) rather than “seed” (HSC) was defective (Knospe and Crosby, 1971) and some workers have found evidence for stromal abnormalities in a minority of AA cases (Hotta *et al.*, 1985). However, cytokine levels are generally increased in most patients (Watari *et al.*, 1989; Das *et al.*, 1992; Lyman *et al.*, 1995; Emmons *et al.*, 1996), suggesting normal marrow stromal function in the majority of patients. Moreover, “cross-over” studies with long-term bone marrow culture have demonstrated normal stromal function in a large majority of AA patients (Marsh *et al.*, 1990).

1.1.5.3 Direct Toxicity as a Causative Mechanism

Myelosuppression may be seen as a predictable, dose-related and transient, response to the administration of radiation or “radiomimetic” cytotoxic drugs or chemicals. However, chronic exposure may result in persistently depressed marrow function, particularly if the therapeutic agent is active against resting, G₀ cells (Hoagland and Gastineau, 1996). Stem cell numbers may be reduced as a result of direct damage to DNA or impaired DNA repair mechanisms (Morley *et al.*, 1978b). Drugs such as benzene produce toxic metabolites whose degradation in the bone marrow is reduced. Such metabolites may covalently bind to DNA and induce apoptosis (Moran *et al.*, 1996). Acetanilide causes AA by direct toxicity in susceptible individuals in whom drug clearance is reduced (Nissen-Druey, 1989). However, most patients in developed countries presenting with AA have had no history of prior exposure to substances with known predictable haemotoxicity (Young and Maciejewski, 1997).

1.1.5.4 Immune Mechanisms

Evidence has mounted in recent years as to the significance of disturbances of immune function in AA (Samson *et al.*, 1974; Ascensão *et al.*, 1976; Benestad, 1979; Nissen, 1991; Young, 1996; Young and Maciejewski, 1997). Early experimental work had shown that marrow hypoplasia could be induced with antibodies (Nettleship, 1942). Following the observation by Floersheim and Ruszkiewicz (1969) that mice made aplastic with dimethyl-myleran could be saved with allogeneic marrow transplantation, provided that they were first conditioned with antilymphocyte (antithymocyte) serum, success was reported in similarly conditioned AA patients (Mathé *et al.*, 1970). As a result, trials of various immunosuppressants, including ALG, antithymocyte globulin (ATG) (together with methylprednisolone, cyclosporin, or cyclophosphamide (CY)) were initiated. Of

these, combinations of ATG with CY and cyclosporin appear favourable (Storb, 1993; Marsh *et al.*, 1999b).

In more than half of the cases of identical twins with AA given marrow from their identical sibling, successful engraftment only occurred after a second transplant, and then only when immunosuppressive conditioning had been given (Champlin *et al.*, 1989). Immunosuppression alone now equals allogeneic BMT as a curative therapy for AA, strongly supporting an immune-mediated mechanism in a proportion of AA cases (Young and Maciejewski, 1997).

Other findings which implicate an immune process in AA include the increased numbers of activated peripheral T cells in AA patients (Zoumbos *et al.*, 1985); also, the finding that mononuclear cells from AA patients inhibit the *in vitro* growth of CFU from normal marrow, and that removal of T cells improves CFU growth (Kagan *et al.*, 1976).

Recently, increased apoptosis of CD34⁺ cells has been demonstrated in AA bone marrow (Philpott *et al.*, 1995; Callera and Falcão, 1997). The mechanism for apoptosis in haemopoietic stem cells appears to be due to the direct effect of nitric oxide (Maciejewski *et al.*, 1995). Induction of nitric oxide synthase expression is itself enhanced through activation of the receptor Fas on CD34⁺ cells (Selleri *et al.*, 1997). Fas expression is increased in lymphocytes in AA (Callera *et al.*, 1998) and is stimulated by IFN γ and TNF α , which are found at increased concentrations in AA (Hinterberger *et al.*, 1988).

Hence a body of evidence exists to suggest that immune mechanisms may be responsible for some cases of drug-induced AA (See 1.1.8.2.3, below; Benestad, 1979; Nissen, 1991; Utrecht, 1992; Young and Alter, 1994).

1.1.6 Treatment of Aplastic Anaemia

A number of factors are considered in relation to treatment, including the age of the patient at presentation; time elapsed since symptoms first appeared; severity of symptoms; blood count and bone marrow findings; previous therapy, including transfusions, and the availability of a compatible sibling donor (Alter and Young, 1998). Nevertheless, the treatment and outcome of the disease is identical in both drug-induced and “idiopathic” AA (Young and Alter, 1994).

Early treatment for AA included splenectomy, therapy with adrenocorticotropic hormone or cortisone (Wolff, 1957), and testosterone (Diamond and Shahidi, 1967). Androgens continue to be used as a second-line treatment (Young and Barrett, 1995). Platelet transfusions, introduced in the late 1950s, have shifted the risk of morbidity from bleeding, to death from overwhelming infection (Alter and Young, 1998). Triple antibiotic therapy with cephalosporins/synthetic penicillins/aminoglycosides or, alternatively a third generation cephalosporin, are now used against fever and neutropenia. Nonetheless, aspergillosis or candidiasis remain frequent causes of death (Alter and Young, 1998).

Haemopoietic growth factors, notably G-CSF and GM-CSF may be useful in stimulating an increase in neutrophil count (Champlin *et al.*, 1989), however, as they cannot correct the underlying stem cell defect, their use in first-line therapy is unwise (Marsh *et al.*, 1994).

Two therapeutic modalities, bone marrow transplantation and immunosuppression, have become the mainstay of treatment for AA over the last three decades (Bacigalupo *et al.*, 1996).

1.1.6.1 Bone Marrow Transplantation

BMT was first attempted in man in 1957 (Thomas *et al.*, 1957). Early experimental findings had demonstrated firstly, that bone marrow infusion protected rodents from the lethal effects of irradiation (Lorenz *et al.*, 1951) and that the immunosuppression with irradiation induced tolerance to the marrow graft, which was functionally active many months later (Congdon *et al.*, 1952; Odell *et al.*, 1957). BMT was first applied in AA patients using marrow from identical twins (i.e., syngeneic marrow), and it was not until 1972 that it could be established that matched allogeneic (i.e., non-identical) transplantation was successful (Thomas *et al.*, 1972). Experimental findings in mice had already shown that syngeneic, and not allogeneic BMT was capable of rescuing animals made aplastic with cytotoxic agents (Floersheim and Elson, 1961). Bone marrow from non-identical siblings is required to be matched with regard to the major histocompatibility loci (HLA) A, B and DR. However, 75 % of AA patients lack a related HLA identical donor and even those phenotypically identical at all three sites are likely to have major or minor mismatches. Recipients of unrelated marrow transplants have a much poorer survival; two major surveys give survival figures of only 29 % and 36 % at two years (Young and Barrett, 1995).

Allogeneic peripheral blood stem cell (PBSC) transplantation is associated with a high rate of engraftment and low incidence of severe graft-versus-host disease (GVHD; Goldman, 1995) and has recently been extended to SAA patients (Redei *et al.*, 1997). The

feasibility of autologous PBSC transplantation in AA has been raised following the finding that sufficient PBSC can sometimes be mobilised with G-CSF in AA patients (Bacigalupo *et al.*, 1993).

1.1.6.2 Immunosuppression

Since it was hypothesised that immunosuppression might in fact be effective against the disease itself (Jeannet *et al.*, 1974), surveys of patients treated with either ALG or BMT have shown that about 45 % of patients with SAA responded to ALG (Young and Barrett, 1995). Cyclosporin is an alternative immunosuppressive agent which inhibits T-cell function (Shevach, 1985) and is effective in about 50 % of patients refractory to ALG or antithymocyte globulin (ATG). Intensive immunosuppression achieved by combining ALG/ATG with cyclosporin has been found to be superior to ALG or ATG alone (Rosenfield *et al.*, 1995) and survival of up to 92 % at three years has been reported when ATG and cyclosporin have been combined with GM-CSF (Young and Barrett, 1995). CY, normally used for immunosuppressive conditioning prior to BMT, may induce long-term remission in SAA when used alone (Brodsky *et al.*, 1996).

1.1.7 Outcome of Aplastic Anaemia

If untreated, most patients with AA die as a result of infection, bleeding or the consequence of severe anaemia (Gordon-Smith, 1989). In a series of 334 cases of AA reviewed in 1957, survival was only 3.3 % (Wolff, 1957). In another series covering 12 years up to 1965, there was 50 % mortality at 5 months (Lewis, 1965). Two subpopulations of AA patients were identified in a later series, one group, approximately 40 %, who survived for less than four months, and the remainder whose mortality rate

was more gradual (Lynch *et al.*, 1975). Prognostic indicators including peripheral blood counts and time to onset of symptoms were strongly associated with survival.

Early mortality in AA (Camitta *et al.*, 1982b) still accounts for approximately 50 % of deaths within the first six months of diagnosis (Alter and Young, 1998). Causes of death other than as a result of failure to resolve AA include marrow graft rejection, GVHD (Ferrara and Deeg, 1991; Bacigalupo, 1999) and clonal syndromes such as paroxysmal nocturnal haemoglobinuria (PNH), pure red cell aplasia (PRCA), MDS, acute leukaemia and solid tumours (Young and Maciejewski, 1997).

1.1.7.1 Late Outcome of Aplastic Anaemia

The actuarial risk of developing PNH, MDS/leukaemia was 57 % at 8 years post immunosuppressive treatment in one survey (Tichelli *et al.*, 1988). A small number of patients (4 %) diagnosed as otherwise classical AA were found to have cytogenetic abnormalities (Appelbaum *et al.*, 1987). In this series of patients, however, 3 % with no cytogenetic abnormalities also went on to develop MDS or AML. In another survey, 72 % of patients with AA exhibited clonal haemopoiesis (van Kamp *et al.*, 1991). These figures support the notion that AA is itself a preleukaemic condition (Marsh and Geary, 1991; Socié, 1996). In transplanted AA patients, the risk of solid tumours is increased. In one series, a rate seven times that of the normal population was seen (Witherspoon *et al.*, 1989). Haematological disease is rare as a late complication of BM transplantation for therapy of AA (Tichelli *et al.*, 1988).

1.1.8 Acquired Aplastic Anaemia

AA is a condition which is associated with a number of rare congenital or inherited conditions (Table 1.4). These conditions are quite heterogeneous, often associated with various developmental defects, but possess, in common, defects to haemopoietic stem cell proliferation, and are curable by bone marrow or stem cell transplantation (Evans, 1989; Storb, 1993; Alter and Young, 1998). However, the majority of AA cases are acquired. Whilst a number of causative agents are associated with the development of AA in man, in about 70 % of patients there is no identifiable causal link. Of the remainder, there are three principal causes: radiation, drugs and chemicals, and infectious agents. Lastly, predisposition to acquired AA has been noted, indicating some constitutional link.

Table 1.4: Epidemiological classification of aplastic anaemia*

Congenital/ inherited	Fanconi's anaemia Dyskeratosis congenita Shwachman-Diamond syndrome Reticular dysgenesis Amegakaryocytic thrombocytopenia Familial aplastic anaemias Preleukemia, myelodysplasia, monosomy 7 Non-haematological syndromes, eg Down's, Dubowitz's, Seckel's
Acquired	Idiopathic Infections: Hepatitis Epstein-Barr virus Human immunodeficiency virus B19 parvovirus Human T lymphotropic virus Mycobacterial infections Toxic Exposure: Radiation Chemicals Drugs Paroxysmal nocturnal haemoglobinuria Transfusional graft versus host disease Orthotopic liver transplant for fulminant hepatitis Pregnancy Eosinophilic fasciitis

*Guinan (1997) and Alter and Young (1998); based on Alter *et al.* (1978).

1.1.8.1 Infections

The majority of infectious agents associated with AA appear to be viral (Cattral *et al.*, 1994; Brown *et al.*, 1994, 1997a, 1997b; Kurtzman and Young, 1989; Weichold *et al.*, 1998). The implication of an immune pathophysiology for AA is consistent with the involvement of, and abnormalities seen in, lymphocytes both in AA patients, and in man and animals infected with viruses (Young, 1997). Viral infection commonly causes transient suppression of erythropoiesis in man, and may be more frequently observed in serial automated reticulocyte counts in patients and clinical trial volunteers (Cavill, 1990; Keisu *et al.*, 1990; Andrews, personal observation). Confident assignment of an aetiological agent in individual cases of AA is usually impossible, but the occasional patient will have obvious antecedent viral illness (Kurtzman and Young, 1989).

1.1.8.2 Paroxysmal Nocturnal Haemoglobinuria

PNH is an acquired, clonal haemopoietic disorder that results from a somatic mutation of the *pig-A* gene in a stem cell of an affected individual. *pig-A* is responsible for the synthesis of glycosylphosphatidylinositol anchors, by which a range of molecules are attached to the cell membrane (Rosse and Ware, 1995), including decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59), which confer protection against complement. A substantial proportion of patients present with pancytopenia and a mild reticulocytosis, and the disease may progress to hyperhaemolysis, bone marrow failure, infection or thrombosis (Rotoli and Luzzato, 1989). The association of PNH and AA may also develop in the opposite direction: 5 - 10 % of AA patients develop PNH as they recover, probably as a result of a selective growth advantage of PNH stem cells over

AA stem cells. Indeed, as many as 50 % of newly diagnosed AA patients manifest the PNH defect, usually in granulocytes (Alter and Young, 1998).

1.1.8.3 Pregnancy

About 60 cases of AA occurring in pregnancy have been reported since Erlich's original description of AA in a pregnant woman (Ehrlich, 1888); the outcome in 50 % of these cases has been fatal, with the most frequent complication being bleeding (Alter and Young, 1998). Pregnancy represents a condition of altered immunity, which may be relevant to the aetiology of AA in these cases. In addition, an imbalance of placental lactogen and erythropoietin, causing increased erythropoiesis, and estrogen levels, which inhibit haemopoiesis, have been implicated in the aetiology of pregnancy-associated AA (Dukes and Goldwasser, 1961, Aitchison *et al.*, 1989). Furthermore, many cases of pregnancy-associated AA spontaneously improve or resolve after induction or delivery, and indeed, marrow hypoplasia may recur with subsequent pregnancies (Fleming, 1968, 1999; Cohen *et al.*, 1993). The association between AA and pregnancy may be coincidental (Oosterkamp *et al.*, 1998); alternatively, bone marrow hypoplasia may be relatively common in pregnancy as it is recognised that patients with pre-existing AA may relapse with the onset of pregnancy (Young and Alter, 1994).

1.1.8.4 Radiation

The induction of myelosuppression is a predictable response to radiation, especially γ -irradiation. The degree of pancytopenia is directly proportional to radiation dose, with lymphocyte count decreasing in a linear fashion; in man, doses exceeding 1.5 - 2 Gy induce bone marrow hypoplasia (Knospe, 1988). The morphology of experimental bone marrow damage by irradiation has been detailed by Congdon (Congdon and Fliedner,

1971) and involves a wide variety of destructive changes. Both stem cells and committed progenitors are susceptible to radiation damage, and in the mouse have been ranked in order of radioresistance as follows: primitive repopulating cells>CFU-S₁₂>CFU-S₇ (Meijne *et al.*, 1991). The relative insensitivity of stem cells to irradiation damage and their very great proliferative potential accounts for the capacity of man and animals to recover from massive single doses of radiation (Young and Alter, 1994). It was originally thought that there was no increase in the incidence of late AA in atomic bomb survivors (Kirshbaum *et al.*, 1971), however, recent re-analysis of survival data suggests that this may not have been the case (Stewart and Kneale, 1999).

Chronic administration of low dose irradiation is associated with haematological abnormalities, including AA. In ankylosing spondilitis patients given radiotherapy (Darby *et al.*, 1987), and in radiologists (Kitabake *et al.*, 1976), the incidence of AA is increased many times over that of the general population.

1.1.8.5 Drugs and Chemicals

AA is associated with a wide range of drugs and chemicals, so much so that almost every class of drug has been cited (Table 1.5; Kaufman *et al.*, 1991; Young and Alter, 1994). The underlying chemical structure of any particular drug is not usually predictive for its propensity to cause AA (Young and Alter, 1994). Furthermore, drugs associated with AA vary in the degree to which aplasia may occur, from those which are highly predictable in their myelotoxicity, such as the antineoplastic agents, to those in which only rare associations have been found (Vincent, 1984; Young, 1988).

Table 1.5: Classification of drugs and chemicals associated with aplastic anaemia*

Predictable¹	<p>Cytotoxic drugs used in cancer chemotherapy:</p> <ul style="list-style-type: none"> Alkylating agents (busulphan, melphalan, cyclophosphamide) Antimetabolites (antifolic compounds, nucleoside analogues) Antimitotics (vincristine, vinblastine, colchicine) Some antibiotics (daunorubicin, adriamycin) <p>Benzene (and less often, benzene-containing chemicals: kerosene, carbon tetrachloride, Stoddard solvent, chlorophenols)</p>
Occasional²	<p>Chloramphenicol</p> <p>Insecticides</p> <p>Antiprotozoals (quinacrine and chloroquine)</p> <p>Nonsteroidal antiinflammatory drugs (phenylbutazone, indomethacin, ibuprofen, sulindac, aspirin)</p> <p>Anticonvulsants (hydantoins, carbamazepine, phenacetin, ethosuximide)</p> <p>Gold, arsenic, other heavy metals (e.g. bismuth, mercury)</p> <p>Sulphonamides:</p> <ul style="list-style-type: none"> Antibiotics Antithyroids (methimazole, methylthiouracil, propylthiouracil) Antidiabetics (tolbutamide, carbutamide, chlorpropamide) Carbonic anhydrase inhibitors (acetazolamide, methazolamide) <p>D-penicillamine</p> <p>Estrogens</p>
Rare³	<p>Antibiotics (streptomycin, tetracycline, ampicillin, mebendazole, sulphonamides, flucytosine)</p> <p>Antihistamines (chlorpheniramine)</p> <p>Antulcerants (cimetidine, ranitidine)</p> <p>Sedatives and tranquillisers (chlorpromazine, prochlorperazine, piperacetazine, chlordiazepoxide, meprobamate, methyprylon)</p> <p>Antiarrhythmics (tocainide)</p> <p>Methyldopa</p> <p>Quinidine</p> <p>Lithium</p> <p>Guanidine</p> <p>Canthaxanthin</p> <p>Potassium perchlorate</p> <p>Thiocyanate</p> <p>Carbimazole</p> <p>Cyanamide</p>

¹ Agents that regularly produce marrow depression as major toxicity in commonly employed doses or normal exposures.

² Agents probably or possibly associated with aplastic anaemia but with a relatively low risk relative to use.

³ Agents more rarely associated with aplastic anaemia.

*Young and Alter (1994)

There are considerable problems in establishing with certainty whether any particular drug is responsible for AA, especially those rarely associated with AA (Table 1.5). AA itself is a relatively rare condition, and historically, information about drug-associated AA was notoriously unreliable. Individual case reports which are not subject to peer review often lacked information about dosage, duration of treatment, concomitant drug use and underlying disease (IAAAS, 1987). The onset of marrow failure is often very difficult to date with accuracy: for example, aplasia may follow benzene exposure only after several years (Snyder *et al.*, 1993). Drug-associated AA may therefore be over-reported. In one review of the literature, attribution of AA to drugs ranged from 86 % of cases to only 7 %, with higher figures from earlier reports and lower figures from the more recent ones (Heimpel, 1996). In general, as criteria for AA are more stringently applied, the association of drugs with AA has reduced to approximately 25 % of cases (Table 1.6).

Table 1.6: Drug and chemical association with aplastic anaemia: clinical series*

Agent	1950-1959	1960-1969	1970-1979	1980-1989
Chloramphenicol	227 (30)	90 (27)	194 (17)	28 (3)
Phenylbutazone	1 (<1)	30 (9)	67 (6)	22 (3)
Sulphonamides	27 (4)	3 (1)	13 (1)	5 (1)
Anticonvulsants	15 (2)	8 (2)	16 (2)	4 (<1)
Gold	0 (0)	3 (1)	12 (1)	12 (2)
Benzene, solvents	24 (6)	14 (4)	37 (3)	21 (3)
Insecticides	9 (1)	29 (9)	15 (1)	11 (1)
Other drugs	118 (16)	26 (8)	169 (13)	60 (7)
Total drug cases	427 (57)	203 (60)	523 (45)	163 (20)
Total cases	756	339	1292	811

*Young and Alter (1994). Numbers in brackets indicate percentage of total cases.

In a recent epidemiological survey, 454 cases of drug-associated AA were matched with 6458 non-drug-related AA controls from a total population of 45 million (Kaufman *et al.*, 1991, 1996). In this survey, only 11 drugs showed a positive association with AA, a much

lower association than for drug-associated agranulocytosis which was assessed in the same study (Kaufman *et al.*, 1996; Table 1.7). Of these 11 drugs, only three (penicillamine, gold and carbamazepine) were prominent, and the increased risk was relatively small. Because of the low rate of prescription of systemic chloramphenicol (CAP), there was insufficient data to provide a meaningful estimate of risk for this drug.

Table 1.7: Aplastic anaemia: use of drugs 29 - 180 days before admission*

	Drug	Cases	Controls	Relative risk ¹	Excess risk ²
Analgesics, NSAIDs	Dipyrone	26	445	0.7 (0.4-1.1)	-
	Other pyrazolones	28	383	0.8 (0.5-1.2)	-
	Salicylates	164	2614	1.1 (0.9-1.5)	-
	Paracetamol	174	2770	1.0 (0.8-1.3)	-
	Phenacetin	19	269	1.1 (0.6-2.0)	-
	Butazones	13	43	3.7 (1.6-8.3)	3.4
	Indomethacin	11	55	2.8 (1.1-6.8)	2.3
	Diclofenac	8	48	3.0 (1.3-7.0)	2.5
	Naproxen	9	61	3.9 (1.6-9.7)	3.6
	Piroxicam	7	28	2.7 (0.8-9.1)	-
Other drugs	Other NSAIDs	15	730	-	-
	Gold	5	4	19 (3.6-97)	23
	Penicillamine	7	2	49 (5.2-464)	60
	Chloramphenicol	5	13	2.7 (0.8-8.7)	-
	Sulphonamides	21	168	2.1 (1.1-4.0)	1.4
	Beta-lactams	35	519	0.9 (0.6-1.4)	-
	Beta-blockers	13	318	1.2 (0.6-2.3)	-
	Triamterene	5	85	1.6 (0.5-4.6)	-
	Quinidine	5	48	1.2 (1.2-3.4)	-
	Furosemide	15	171	2.8 (1.4-5.6)	2.2
	Carbamazepine	4	12	13 (3.3-54)	15
	Allopurinol	8	38	4.6 (1.7-12)	4.5
Total cases	Corticosteroids	19	100	4.0 (2.0-7.7)	3.7
		454	6458		

*Kaufman *et al.* (1996). ¹95% confidence intervals. ²Number of cases per million users per 5 month period.

1.1.8.5.1 Cancer Chemotherapeutic Agents

The dose-limiting toxicity of many anticancer drugs is myelosuppression. Nevertheless, increasing dose is not necessarily correlated with late effects or irreversible stem cell damage (Young and Alter, 1994). Amongst the most severely toxic of these drugs are the alkylating agents (Table 1.8) of which busulphan (BU) is notorious for its ability to cause permanent marrow aplasia (Weatherall *et al.*, 1969; Stuart *et al.*, 1976).

Table 1.8: Levels of severity of myelosuppressive effects of antineoplastic drugs*

Absent to mild	Moderate	Severe
Vincristine	Vinblastine	Alkylating agents
Bleomycin	Actinomycin D	(e.g. nitrosoureas,
L-asparaginase	Epipodophyllotoxins	nitrogen mustards,
Hormones	Cisplatin	alkyl sulphonates)
Tamoxifen	Purine analogues	Anthracyclines
	Methotrexate	Anthroquinones
	Hexamethylmelanine	Mitomycin C
	Procarbazine	Pyrimidine analogues
		Hydroxyurea

*Gale (1988).

There is a wealth of evidence that other chemotherapeutic agents cause long-term marrow damage in man and experimental animals (Botnick *et al.*, 1979; Morley, 1980; Testa and Gale, 1988; Down *et al.*, 1989; Hoagland and Gastineau, 1996). Stromal haemopoiesis, CFU-GM number and proliferation are reduced following chemotherapy (Miller and Weiner, 1988; Parmentier *et al.*, 1988; Testa *et al.*, 1988); the damage to CFU-GM does not improve with time (Chang *et al.*, 1990). Other evidence supporting permanent marrow impairment includes persistent chromosomal aberrations, irreversible damage to other organs, and the risk of secondary leukaemias (Lohrmann and Schreml, 1988). Cumulative toxicity to cytotoxic therapy may develop, with greater depression of

leucocyte or platelet count, or a delayed or incomplete response, especially to nitrosoureas or mitomycin C.

Dosage, regimen, and combination with, either concurrently, or subsequent to, other chemotherapeutic agents or radiotherapy, may enhance the extent and duration of the myelosuppression. Amongst those drugs causing severe myelosuppression, the rate of myelosuppression varies, depending on the affected cell lineage and its developmental stage. Thus hydroxyurea causes an immediate and marked decrease of granulocytes, whose lifespan is 24 - 48 h, whereas nitrosoureas appear to be selectively active against early stem cells and their suppressive effect may not be seen until 6 - 8 weeks (Gale, 1988). The effects of BU (an alkyl sulphonate) on stem cells may be latent and not evident for months or years, whilst other alkylating agents such as melphelan and CY act on committed and maturing cell populations. Lymphocytes are particularly sensitive to the effects of nitrogen mustards, which makes this group particularly effective as immunosuppressants. For example, with methchlorethamine, lymphocytopenia becomes evident after 24 hours, increasing in severity for 6 - 8 weeks, whilst granulocytopenia develops over 1 - 3 weeks (Calabresi and Parks, 1985).

1.1.8.5.2 Benzene and Other Hydrocarbons

Benzene, a commonly used industrial solvent, and a component of petrol and cigarette smoke, is the most firmly established causative agent in AA. In addition to AA, a variety of haematological malignancies, including MDS and acute leukaemia, CML and multiple myeloma have been reported in association with benzene exposure (Snyder *et al.*, 1993; Snyder & Kalf, 1994). Metabolism of benzene, which also occurs within the bone marrow, is necessary for toxicity, with the production of phenolic metabolites. Benzene

and its metabolites block cell division at G₂/M phase, inhibit microtubule assembly and induce aneuploidy in cultured human lymphocytes. An important metabolite of benzene, hydroquinone, known to concentrate in bone marrow, is implicated in leukaemogenesis via enhanced myeloid progenitor cell responsiveness to GM-CSF (Irons *et al.*, 1992).

Aplasia following benzene exposure is probably 5-6 times more common than acute leukaemia, but the literature on benzene and haematological abnormalities is inconsistent, so much so that it is impossible accurately to state the real incidence of such toxicity (Goldstein, 1977; Jacobs, 1977). It is probable that the past incidence of benzene-associated AA is under-reported. Early US studies suggest that the risk of AA is about 3 - 4 % in workers routinely exposed to >300 ppm, and about half of those who were exposed to 100 ppm showed some blood count depression (Young and Alter, 1994).

The evidence linking other chemicals to AA in man is more circumstantial. Chemicals used in industry and agriculture associated with AA include the aromatic hydrocarbons chlordane and lindane, and organochlorine pesticides (Loge, 1965; Rugman and Cosstick, 1990), pentachlorophenol (Roberts, 1990) and trinitrotoluene (Hathaway, 1977). However, some studies of AA in agricultural populations do not implicate the use of pesticides (Issagrasil *et al.*, 1996).

1.1.8.5.3 Chloramphenicol

CAP was first introduced into clinical practice in 1948, and the first report of AA associated with its use was in 1950 (Rich *et al.*, 1950) and was the most frequently cited drug in surveys of drug-associated AA for many years (Table 1.6, 1.9)

Table 1.9: Epidemiological studies of chloramphenicol and aplastic anaemia*

Area	Date	Percentage of Drug Cases	Percentage of Total Cases
US	1949-52		47
California	1957-61	78	29
California	1963-64	59	17
Sweden	1966-71	17	6
Israel	1961-65	56	25
Japan	1958-74	30	3
Japan	1973	45	8
Europe-Israel	1980-86	0	0

*Young and Alter (1994).

It was not until the mid 1960s that sales of CAP began to fall and warnings to prescribe CAP only for life-threatening illnesses were printed on packaging in the US. In one recent survey, due to the reduced prescription of CAP, there was insufficient data to detect any excess risk of AA with the drug (Kaufman *et al.*, 1996; Table 1.7).

Estimates for the risk of AA after a course of CAP treatment range from 1/20,000 to 1/800,000 with the figure of 1/60,000 gaining the most credence (Smick *et al.*, 1964). These figures are based on an average therapeutic course of 4 g and assume causality in all cited cases. However, a relationship between CAP dosage and AA has never been clearly established. Some surveys indicate a relationship with AA and intermittent courses of CAP (Wallerstein, 1969), whilst others pointed out that higher dosages (>40 g) tended to be associated with reversible marrow depression (Yunis and Bloomberg, 1964; Meyler *et al.*, 1972). Because no no-effect level for CAP and AA could be established, it was not possible to give an assurance that residues in foods of animal origin would be safe for sensitive subjects, and no Acceptable Daily Intake, or permission for extralabel

use in food animals, could be allocated for CAP (FAO/WHO Expert Committee on Food Additives, 1988; Payne *et al.*, 1999).

CAP continues to be widely prescribed in the developed world for topical application for ophthalmic use. In spite of recent concerns, there appears to be little evidence for systemic exposure by this route, and in spite of more than 200 million ocular CAP products being dispensed in the UK in the decade preceding 1996, only 11 cases of suspected CAP-induced blood dyscrasias (all of which were non-fatal) were reported between 1966 and 1995 (McGhee and Anastas, 1996). No cases of ocular CAP-induced AA were reported in either of two IAAAS studies which encompassed 426 AA cases from a population of more than 40 million (Wilholm *et al.*, 1998)

CAP-associated AA was seen predominantly (62 % of cases) in females (Chaplin, 1986b) with peaks in both sexes in the 6 - 10 year-old and 31 - 50 year-old groups (Yunis and Bloomberg, 1964). No racial or ethnic factors appear significant in the predisposition to CAP-associated AA (Chaplin, 1986b). There is some evidence for genetic susceptibility in that CAP-associated AA can occur in identical twins (Best, 1967; Nagao and Mauer, 1969).

1.1.8.5.4 Nonsteroidal Antiinflammatory Agents

The most prominent drug to be associated with AA amongst NSAIDS is phenylbutazone (PBZ), which appears to cause AA with almost the same frequency as CAP (Table 1.6, 1.7). PBZ, like CAP, also causes temporary and reversible marrow suppression, but the major hazard associated with this drug is agranulocytosis (McCarthy and Chalmers 1964; Chaplin, 1986a; IAAAS, 1986). Many early cases of PBZ were associated with very high

dosages and, as with CAP, AA may be related to decreased drug clearance (Cunningham *et al.*, 1974). Other NSAIDS have been identified with at least as high risk factors for AA as PBZ including indomethacin, diclofenac and naproxen (Kaufman *et al.*, 1996; Table 1.7). Aspirin and ibuprofen are also implicated in the aetiology of AA, and patients may develop a second episode of marrow suppression after exposure to a different NSAID (Young and Alter, 1994). Benoxaprofen (Opren) was associated with a number of cases of AA before its withdrawal from the market on the basis of its toxicity (Gordon-Smith, 1989).

1.1.8.5.5 Other Drugs

Anticonvulsants, especially the hydantoins and carbamazepine, have been particularly associated with AA, although the frequency of AA with the latter may have suffered from overreporting in the past (Young and Alter, 1994). Heavy metals, in particular gold and its salts, are strongly associated with AA. Gold therapy was associated with the highest (with penicillamine) excess risk of AA (Kaufman *et al.*, 1991; 1996). The recreational drug ecstasy has been reported to cause AA (Clark and Butt, 1997).

1.1.8.5.6 Drugs Recently Associated with Aplastic Anaemia

In recent years, a number of drugs have been taken off the market after reports of AA associated with their administration. Remoxipride, an antipsychotic, was marketed by Astra in Europe in 1991. After clinical trials in 50,000 patients, eight cases of AA were found to have occurred and the drug was withdrawn in 1993 (Philpott *et al.*, 1993). This experience and the realisation that preclinical testing could not predict this kind of toxicity prompted Astra to organise and fund a conference, held in Sweden in 1995 to examine all aspects of drug-associated AA. The proceedings were published as a

supplement of the European Journal of Haematology (Suppl 60 Volume 57, 1996). In 1997 Astra funded a \$640,000 two-year programme to elucidate mechanisms underling remoxipride-induced AA.

Felbamate, an anticonvulsant, manufactured by Carter-Wallace, was first approved for market in August 1993. By 1997, 23 cases of AA had been reported in association with its use (Kaufman *et al.*, 1997). An evaluation of these cases concluded that felbamate was the most likely cause of 11 of them and possibly the cause of another 9, producing an estimate of risk of AA of 127 per million population per year, about 50 times the spontaneous rate. In August 1994 felbamate was withdrawn in the US and South American countries after trials involving over 100,000 patients, but has been relaunched under licence by Schering Plough in Europe and other countries in 1996 for certain indications.

Ticlopidine, a platelet anti-aggregant, was first launched by Sanofi in France in 1978. Registration for use in the US only occurred in 1992, specifically for stroke prevention in patients intolerant to aspirin. Since 1992, reports of AA associated with the use of ticlopidine began to appear and by 1998 the total number in the literature was nineteen (Yeh *et al.*, 1998; Love *et al.*, 1998). Nevertheless, this drug continues to be used for these specific indications.

Clozapine and mianserin, both antipsychotic drugs, and dipyrone, an NSAID, have been strongly associated with agranulocytosis and occasional reports of AA (Chaplin, 1986a; Kaufman *et al.*, 1996; der Klauw *et al.*, 1998).

1.2 Aplastic Anaemia in Animals

1.2.1 Naturally-occurring Aplastic Anaemia

Female ferrets in protracted estrus commonly develop AA. Of a group of 20 ferrets observed during estrus, all became thrombocytopenic, just under half became moderately anaemic and eight died or were killed when they became profoundly thrombocytopenic (Sherrill and Gorman, 1985). AA in the ferret is due to elevated levels of estrogen (Hart, 1990). Malignancies such as Sertoli cell tumours (Sherding *et al.*, 1981) and testicular interstitial cell tumours (Suess *et al.*, 1992) which cause elevated estrogen levels have been associated with AA in the dog.

Idiopathic AA has been reported in cats (Weiss and Evanson, 2000), and AA in cats may develop following infection with feline panleucopenia virus, which has a relative specificity for the myeloid series (Kurtzman and Young, 1989). Feline leukaemia virus-Sarma-subgroup C infection causes PRCA in cats associated with a pronounced depletion of BFU-E *in vitro* (Onions *et al.*, 1982; Dornsife *et al.*, 1989). This anaemia is nonregenerative and often described as “aplastic” anaemia, however the myeloid lineage is relatively spared (Hoover *et al.*, 1974). Feline immunodeficiency virus causes a range of symptoms in cats comparable to human acquired immunodeficiency syndrome (AIDS) and AIDS-related complex; cytopenias of all lineages are seen in symptomatic animals (Shelton *et al.*, 1990). Bacterial sepsis may result in aplasia in cats (Weiss and Evanson, 2000). Chicken anaemia agent, the virus causing chicken infectious anaemia, is responsible for several disease entities including AA. Yellow fatty marrow and thymic atrophy are the most consistent features. (Pope, 1991).

Drugs administered in veterinary practice, as in human clinical practice, have been implicated in AA in animals (Lavoie *et al.*, 1987; Weiss *et al.*, 1999; Weiss and Evanson, 2000). In one series, estrodiol, NSAIDs (phenylbutazone and meclofenamic acid), antibacterials and antihelminthics (trimethoprim/sulphadiazine and fenbendazole) and quinidine were identified as causative agents of marrow aplasia (Weiss and Klausner, 1990). Therapeutically-administered estrogens have been associated with AA in dogs (van Kruinigen and Friedland, 1987)

1.2.2 Experimentally-induced Aplastic Anaemia

Experimental models of AA have been developed from the known and supposed agents inducing marrow hypoplasia and AA in man, including infective agents, radiation, drugs and chemicals, estrogens and antibodies (Alter *et al.*, 1978; Haak, 1980; Vincent, 1984). Genetically modified strains of laboratory animals with specific haemopoietic defects of interest in the study of AA have also been described (Harrison, 1979; Ryffel, 1997).

1.2.2.1 Infective Agents and Immunosuppression

Nettleship (1942) administered guinea-pig anti-bone marrow serum to rabbits. Anaemia and bone marrow changes, including fatty replacement, necrosis and fibrosis, were evident in animals up to 2 months post treatment. Based on findings by Barnes and Mole (1967) two groups subsequently developed an immunologically-mediated model of mouse AA in which sublethally-irradiated hosts were injected with mouse lymph node cells (Kubota *et al.*, 1978; Knospe *et al.*, 1983). AA was found to be T-cell mediated in this model (Chiu and Knospe, 1987) and there was evidence of both stem cell and marrow stromal injury (Knospe *et al.*, 1994). This mouse model has recently been adapted, using non-irradiated hosts, to demonstrate the efficacy of immunosuppression and anti-IFN γ in ameliorating the severity of AA (Wolk *et al.*, 1998). Murine hepatitis

virus has been employed as an agent for the induction of AA (Camillo *et al.*, 1982a) and perforin-deficient mice infected with lymphocytic choriomeningitis virus develop AA which was prevented by depleting CD8⁺ T cells (Binder *et al.*, 1998). Lastly, lymphocytes from dogs with tropical canine pancytopenia, caused by *Ehrlichia canis* infection, were cytotoxic to autologous monocytes (Haak, 1980) suggesting an immune-mediated basis in this model of AA.

1.2.2.2 Radiation

Late AA following irradiation has been demonstrated in a variety of species including mice, rats, rabbits and dogs (Barnes and Mole, 1967; Ludwig *et al.*, 1967; Fliedner *et al.*, 1986; Seed *et al.*, 1993). In mice which did not develop leukaemia after irradiation, preleukaemic changes to the marrow and other organs included a long-lasting suppression of erythroid precursors (Ludwig *et al.*, 1967). Mice given ⁸⁹Sr developed bone marrow aplasia, but erythropoiesis was maintained by splenic output (Klassen *et al.*, 1972). Rabbits given ³²P developed AA and were used to demonstrate the efficacy of bone marrow transplantation following conditioning with antilymphocyte serum (ALS; Speck and Kissling, 1973). Permanent aplasia may be induced in rats irradiated at 40 Gy (Knospe, 1988). At levels of between 20 and 100 Gy, extensive injury to marrow sinusoids occurs in the first 24-48 hours after irradiation, and aplasia ensues 2 d later. Thereafter, spontaneous repair and regeneration leads to normal or increased cellularity by 14 d, followed by a secondary wave of aplasia 1-3 months after irradiation. Regeneration of marrow sinusoids and haemopoiesis occurs after 6-12 months after 20 Gy, whereas at 40-100 Gy, haemopoiesis does not recover. At 60-100 Gy, fibrosis occurs progressively. In dogs, modelling of radioresistance shows the presence of distinctive

subgroups: low resistance is associated with the development of AA, and higher resistance with the development of myeloproliferative disorder (Seed *et al.*, 1993).

1.2.2.3 Drugs and Chemicals

1.2.2.3.1 Benzene

Rabbits treated ip with 0.3 ml benzene/kg/d developed pancytopenia within 1 to 9 weeks (Moeschlin and Speck, 1967). Marrows were mostly hypoplastic after this time, but where the erythroid series was represented, radiolabelling studies indicated a maturation block at the level of the early normoblast. Administration by inhalation of 302 ppm benzene for 26 weeks to CD₁ mice resulted in moderate anaemia, marked lymphocytopenia, and reduced marrow and splenic cellularity (Green *et al.*, 1981).

Similar models have been described for CBA strain mice (Farris *et al.*, 1993), B6C3F₁ mice (Farris *et al.*, 1997) and rats (Barrera Escoria *et al.*, 1997). The leucopenic effect of benzene administration in rats was largely abolished by prior phenobarbital treatment (Gill *et al.*, 1979).

1.2.2.3.2 Busulphan and Other Cytotoxic Agents

Many animal models of myelotoxicity with cytotoxic agents have been described (e.g., Berman *et al.*, 1969; Trainor and Morley, 1976; Trainor *et al.*, 1979; Boggs and Boggs, 1980; Schurig *et al.*, 1986; Berger, 1987; Molineux *et al.*, 1987; Kato *et al.*, 1988). Early attempts were made to establish models of AA in rabbits and mice using BU alone and in combination with other drugs (Lu *et al.*, 1956/7; Speck and Moeschlin, 1968). Late marrow aplasia in mice following BU was described by Morley and Blake (1974a). The toxicity of BU to stem cells, and their response to a variety of granulopoietic (Udupa *et*

al., 1972; Delmonte, 1978; Boggs *et al.*, 1980) and erythropoietic stimuli (Morley *et al.*, 1976a; Jelkmann and Bauer, 1980) has been quantified. Similarly, the effect of BU on the marrow stroma (Hays *et al.*, 1982; Wathen *et al.*, 1982; Qi *et al.*, 1991), and the immunosuppressive properties of BU (Addison, 1973; Cottney *et al.*, 1980) have been widely studied. Aplastic rats have proved a useful preclinical model for bone marrow transplantation, first using BU alone and later in combination with CY (Santos and Tutschka, 1974; Tutschka *et al.*, 1980). The BU-treated rabbit has been proposed as a model of human AA (den Ottolander *et al.*, 1982) and the proliferative defect in stem cells has been measured 6.5 years after BU treatment in cats (Abkowitz *et al.*, 1993).

1.2.2.3.3 Chloramphenicol

Administration of CAP alone has been reported to cause AA in Holstein calves (Krishna *et al.*, 1981). When given orally at 100mg/kg for 10 d, reduction of marrow cellularity and morphological abnormalities including vacuolation of erythroid precursors were still evident six weeks post dosing. The peripheral blood picture was not affected, except for slight lymphocytosis. However, no effect on peripheral blood or bone marrow was observed after intravenous (iv) administration of up to 150 mg/kg (six twelve-hourly doses of 25 mg/kg) in neonatal Holstein calves (Burrows *et al.*, 1984). Whilst CAP usually induced a reversible anaemia in ducks, it was noted that persistent reticulocytopenia could occur, with the ultimate death of the bird (Rigdon *et al.*, 1954).

1.2.2.3.4 Estrogens

Estrogens appear to exert a bifunctional effect on haemopoietic tissue, greatly stimulating the proliferation of chicken erythroid progenitors and delaying their maturation (Krijanovski and Sieff, 1997). In the ferret and dog, prior to the development of aplasia,

an initial hyperplastic, leukaemoid, phase is seen in response to ethinyl oestradiol, suggesting a stimulatory effect on committed precursors (Capel-Edwards *et al.*, 1971; Hart, 1985; Sherrill and Gorman, 1985). Long-term administration of hydroxy-estrin benzoate or equilin benzoate led to complete replacement of the marrow cavities of long bones of mice and the appearance of lymphoid tumours (Gardner and Pfeiffer, 1943). Splenic haemopoiesis was seen concurrently with marrow aplasia in mice following oestradiol benzoate administration, suggesting a specific effect on the bone marrow microenvironment (Anagnostou *et al.*, 1976).

1.2.2.3.5 Other Drugs and Chemicals

Aykac *et al.*, (1976) reported that the solvent trichlorethylene given orally to calves at 200 mg/kg for three days resulted in partial marrow aplasia. *S*-(1,2-dichlorovinyl)-L-cysteine, identified as the agent responsible for fatal haemorrhage in cattle fed trichlorethylene-extracted soybean oil meal (McKinney *et al.*, 1957), caused complete aplasia after a single iv dose of 3 or 4 mg/kg (Schultze *et al.*, 1959; Aykac *et al.*, 1976) or after 10 daily i.v. doses of 0.4 mg/kg when given to Guernsey or Friesian calves (Lock *et al.*, 1996).

Antiviral compounds which inhibit viral nucleic acid synthesis, such as nucleoside analogues, can cause aplasia in laboratory animals at very high doses (Andrews, unpublished observations), and their dose-limiting toxicity in man is myelosuppression (Richman *et al.*, 1987; Morris, 1994). However, azidothymidine (AZT), 2', 3'-dideoxycytidine and zidovudine, when administered at levels significantly higher than the recommended dose for use in man, induce a macrocytic anaemia with reticulocytopenia in mice and rats. Mice are more susceptible than rats to haemotoxicity by these agents,

and may also develop marrow hypocellularity; however, all such effects are reversible (Thompson *et al.*, 1991; Ayers *et al.*, 1996).

1.2.2.4 Genetic Models of Aplastic Anaemia

W/W^v and Sl/Sl^d mice are models for complementary haemopoietic defects at the level of the haemopoietic stem cell and are typified by macrocytic anaemia, thymic hypoplasia and lymphoma/leukaemia (Harrison, 1979). In the W/W^v mouse, the marrow stroma is normal but stem cells lack the receptor for SCF, or c-kit receptor (Altus *et al.*, 1971; Huang *et al.*, 1990). Stem cells of Sl/Sl^d mice on the other hand, are normal but the microenvironment does not produce SCF. In cross-transplantation experiments, Sl/Sl^d (and normal) cells correct the haemopoietic defect in W/W^v mice, but W/W^v (and normal) stem cells will not grow in Sl/Sl^d mice (Broxmeyer *et al.*, 1991). The defects in both strains induce lack of sensitivity to 3,4-epoxybutene (EB), a metabolite of the solvent 1,3 butadiene. However, EB induces a similar spectrum of pathological changes in normal B6C3F₁ mice to those seen spontaneously in W/W^v and Sl/Sl^d mice. It is inferred that in W/W^v and Sl/Sl^d mice there is a net functional deficiency in a primitive stem cell population responsive to growth factors controlling early proliferation and differentiation, and that this population is susceptible to EB toxicity in normal mice (Irons *et al.*, 1993). Nevertheless, ip administration of CAP to W/W^v mice was ineffective in reducing femoral CFU-C and peripheral cell counts (Nagai and Kanamura, 1977).

Ah^d/Ah^d mice are susceptible to the induction of aryl hydrocarbon hydroxylase activity and numerous associated cytochrome P450 enzymes by polycyclic aromatic hydrocarbons (Nebert *et al.*, 1977). These mice develop AA after administration of benzo[a]pyrene in a

dose-dependent manner, but with a latency period that is inversely proportional to dose. CAP toxicity is not, however, associated with the Ah locus (Nebert, 1981).

A mouse model of Fanconi anaemia has been generated which displays a deficiency in the Fanconi anaemia group C complementation gene. These so-called *Fac*-/- mice, whilst not exhibiting any demonstrable changes in peripheral cell counts, or any tendency to pancytopenia, as occurs in FA patients, are extremely sensitive to the DNA-cross-linking agents mitomycin C (MMC) and diepoxybutane. A single ip dose of 1mg/kg MMC resulted in peripheral pancytopenia and death within 10 d. Weekly doses of 0.3 mg MMC/kg caused progressive pancytopenia over 5-8 weeks with marrow aplasia; marked reductions in committed precursors in marrow and spleen (CFC) and more primitive long-term culture initiating cells (LTC-IC) were detectable after one week (Carreau *et al.*, 1998)

Mice lacking a wide range of haemopoietic cytokines or their receptors (knockout mice) have been described (Ryffel, 1997). In general, single cytokine knockouts do not cause aplasia, but affect the proliferation and maturation of specific cell lines, thus, for example, deletion of the G-CSF gene results in chronic neutropenia which is correctable by the administration of G-CSF. Inactivation of the IL-2 receptor γ -chain gene results in a condition similar to X-linked severe combined immunodeficiency in man; this knockout mouse has proved to be a useful host in the study of transplanted tissue, including haemopoietic cells.

1.3 Chloramphenicol

1.3.1 Structure, Mode of Action, Therapeutic Indications

CAP, (D-(-)-threo-1-p-nitrophenyl-2-dichloro acetamido 1-3-propanediol), is a highly active broad-spectrum antibiotic originally isolated from the actinomycete *Steptomyces venezuelae* in 1947 and was first marketed by Parke-Davis in 1949.

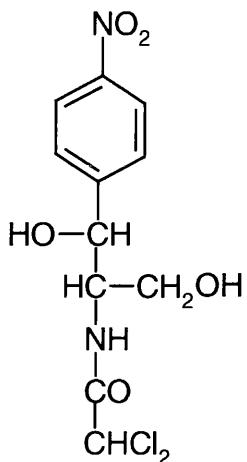


Figure 1.1: Chemical structure of CAP

As a prescription-only medicine in the UK, it is now reserved for serious and life-threatening infections such as meningitis, typhoid fever and other severe salmonella infections (Holt *et al.*, 1993, Parfitt, 1999). It is the only known antibiotic with a nitrobenzene moiety; a propanediol moiety is critical for its antibacterial activity which is due to inhibition of bacterial protein synthesis (Holt *et al.*, 1993). The usual dose for adults and children is 50 mg/kg daily in divided doses every six hours, although up to 100 mg/kg/day may be given in severe infections due to moderately resistant organisms (Parfitt, 1999). For full-term neonates under 7d of age, 25 mg/kg/day is recommended (Holt *et al.*, 1993). At normal therapeutic levels, CAP is bacteriostatic. Sensitive organisms are completely inhibited at concentrations of 10 mg/L (Yunis and Bloomberg,

1964), and a single (oral) dose of 2g CAP will produce a serum concentration of 20 - 40 mg/L. Recommended peak-trough serum concentrations are between 20 and 5 mg/L (Parfitt, 1999). CAP is completely absorbed from the gastrointestinal tract, with a bioavailability of 76 - 93 %, and 75 - 90 % is excreted into the urine over a 24 h period (Ambrose, 1984; Holt *et al.*, 1993). The major route of excretion in man is by conjugation with glucuronic acid, but the -glycol alcohol, -aldehyde, and -oxamic acid derivatives, and CAP base have also been detected in urine of treated patients (Holt *et al.*, 1993, 1995); up to 15 % is excreted as unconjugated CAP (Ambrose, 1984).

1.3.2 Chloramphenicol Toxicity

CAP is haemotoxic in two quite separate ways (Yunis, 1973). Firstly, CAP causes a predictable, dose-dependent myelosuppression, primarily affecting erythropoiesis, although thrombopoiesis and granulopoiesis may also be affected. In general, dose levels associated with this form of toxicity have been relatively high and given over a short time period. Myelosuppression is reversible upon withdrawal of CAP (Volini *et al.*, 1950; Krakoff *et al.*, 1955; Saidi *et al.*, 1961; Jiji *et al.*, 1963; Scott *et al.*, 1965). Secondly, AA may develop in some patients. This form of toxicity is rare and unpredictable and may occur weeks or months after CAP therapy. There is no clear relationship to dose, but intermittent dosing often precedes the development of AA, which is unrelated to prior reversible haemotoxicity (Yunis and Bloomberg, 1964; Wallerstein *et al.*, 1969). These two forms of toxicity have been labelled Type I (reversible marrow suppression) and Type II (AA) reactions (Yunis and Bloomberg, 1964), or Type A and B reactions respectively (Chaplin, 1986a).

1.3.2.1 Type A Haemotoxicity

The mechanism underlying the reversible haemotoxicity of CAP is thought to relate to CAP's ability to inhibit mitochondrial protein synthesis (Yunis *et al.*, 1970).

Erythropoiesis is particularly sensitive to inhibition by CAP, both *in vivo* and *in vitro*, and has been shown to be proportional to mitochondrial damage (Saidi *et al.*, 1961).

The development of reticulocytopenia, occurring 2 d after CAP administration, is consistent with maturation block and vacuolation at the early normoblast stage, whilst the more mature erythroid cells are able to progress to maturity (Firkin, 1972). Haem synthesis is inhibited as a result of decreased activity of δ -aminolevulinic acid synthetase and ferrochelatase (Manyan *et al.*, 1972; Rosenberg and Marcus, 1974). Iron accumulation in the mitochondria of late normoblasts (sideroblastic change) is demonstrable in the bone marrow (Yunis and Salem, 1980), and elevations of serum iron concentration and total iron-binding capacity are seen (Rubin *et al.*, 1958; Scott *et al.*, 1965). Reticulocyte haem and globin production are both inhibited *in vitro* with CAP incubation (Gruenspecht *et al.*, 1979). Ultrastructural changes in early erythroid cells of mouse splenic colonies include increased numbers of mitochondria, which are swollen, and contain an electron lucent matrix and paucity of cristae (Miura *et al.*, 1980). Others have reported vacuolation of the cytoplasm and nucleus of erythroblasts, as well as myeloid and lymphoid precursors and other cells (Krakoff *et al.*, 1955), and the presence of lipid inclusions in vacuoles (Rosenbach *et al.*, 1960; Schober *et al.*, 1972; Skinnider and Ghadially, 1976).

CAP administration caused an increase in the number of CFU-S and CFU-GM in mice when a serum concentration of between 20 and 40 mg/L was maintained for 3 - 5 d

(Firkin *et al.*, 1974). However, 50 % inhibition of murine CFU-GM *in vitro* was seen at between 1 - 10 mg/L, and at 14 mg/L with human colonies (Morley *et al.*, 1974a). CFU-GM and CFU-E showed similar sensitivity to CAP in both man and mouse at concentrations of 10 mg/L and greater, but the more primitive BFU-E were inhibited at around 5 mg/L (Hara *et al.*, 1978). Mouse strains show variable sensitivity to *in vitro* CAP-induced CFU-E inhibition. At comparable concentrations, approximately 30 % or 90 % inhibition of CFU-E growth may be seen, according to mouse strain (Miller *et al.*, 1978). At approximately 1 mg/L, CAP stimulated CFU-GM production in man, and caused inhibition at higher concentrations, whereas CAPS inhibited CFU-GM growth at all concentrations from 0.01 to 50 mg/L (Bostrom *et al.*, 1986). Bone marrow depression in man was associated with plasma CAP levels of 25 mg/L and above (Scott *et al.*, 1965).

1.3.2.2 Type B Haemotoxicity

The underlying mechanism for CAP-associated AA has not been determined (Young and Maciejewski, 1997). However, there is limited evidence for a genetic predisposition to CAP susceptibility. CAP has been shown to inhibit DNA synthesis at lower concentrations in marrow cells from patients, and from relatives of patients with CAP-associated AA, than in marrow from normal individuals (Yunis, 1973). Furthermore, the inhibition of erythroid colony growth by CAP shows wide variation in different mouse strains (Miller *et al.*, 1978). Case reports of CAP-induced AA in identical twins (Best, 1967; Nagao and Mauer, 1969) provide rather more direct support for this hypothesis.

The nitroso- derivative of CAP (R-N=O) is a putative metabolite which is more toxic than the parent compound, causing inhibition of DNA synthesis, of human CFU-GM and mouse CFU-S, at much lower concentrations than CAP itself (Yunis *et al.*, 1980b).

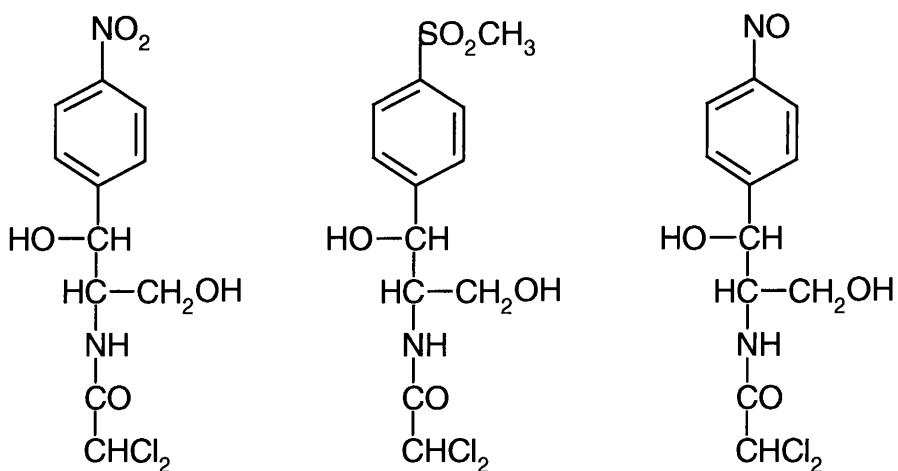


Figure 1.2: Chemical structure of (from left to right): CAP, TAP and nitroso-CAP

Nitroso-CAP is more suppressive of murine splenic lymphocyte function than CAP alone (Pazdernik and Corbett, 1980). Nitroso-CAP is derived from the $-\text{NO}_2$ moiety of CAP, which is absent from the analogue thiamphenicol (TAP), in which a methylsulphonyl group is substituted (Yunis, 1978). It was originally thought that TAP was not associated with AA (Keiser, 1974; Ferrari, 1984), but more recent epidemiology suggests that AA does occur with TAP at a rate comparable with CAP (Young and Alter, 1994); nevertheless, use of TAP in beef cattle is permitted in the United States (Payne *et al.*, 1999). Whilst nitroso-CAP does not form in bone marrow and has a very short half-life, auto-oxidising to hydroxylamino-CAP, CAP-amine has been identified in the serum of some CAP-treated patients, implying that the haemotoxic intermediate had been present (Holt *et al.*, 1993). Dehydro-CAP, a toxic bacterial metabolite potentially generated by intestinal flora, has also been proposed as a potential agent in CAP-associated AA (Jimenez *et al.*, 1987).

1.3.2.3 Toxicity in Man

In man, CAP causes acute, reversible anaemia, granulocytopenia and thrombocytopenia (Type A haemotoxicity) with maturation arrest of both erythroid and granulocyte precursors in patients (Volini *et al.*, 1950; Saidi *et al.*, 1961) and healthy volunteers (Jiji *et al.*, 1963; Scott *et al.*, 1965). Reduction in iron metabolism (Rubin *et al.*, 1958), vacuolation of erythroid precursors (Rosenbach *et al.*, 1960) and damage to mitochondria (Skinnider and Ghadially, 1976) have been described.

The first report of AA (Type B haemotoxicity) in man was in 1950 (Rich *et al.*, 1950), prompting surveys of AA deaths and their relationship to CAP in California between 1957 and 1961 (Smick *et al.*, 1964) and 1963-4 (Wallerstein *et al.*, 1969). These surveys estimated a risk of death from AA of between 1/132,900 and 1/14,500 of patients treated with CAP (Wallerstein *et al.*, 1969) and 1/60,000 (Smick *et al.*, 1964). Myeloblastic leukaemia development in CAP-associated AA patients has also been suggested as a consequence of CAP administration (Brauer and Damashek, 1967).

CAP causes severe, life-threatening toxicity at serum concentrations above 40 mg/L in neonates. The so-called grey baby syndrome is characterised by abdominal distension, vomiting, metabolic acidosis, progressive pallid cyanosis, irregular respiration, hypothermia, hypotension, and vasomotor collapse. All of these features may be due to the attack of oxygen free radicals, possibly via the metabolites of CAP (Holt *et al.*, 1993).

CAP has been reported to cause hearing loss, particularly if administered topically (Gargye and Dutta, 1959). CAP-induced optic neuritis, pseudomembranous colitis and

haemolysis in glucose-6-phosphate dehydrogenase (G6PDH) deficient subjects have also been reported (Holt *et al.*, 1993).

1.3.2.4 Toxicity in Animals

Gavage dosing of up to 750 mg/kg/day to cynomolgus monkeys for 15 months did not produce any haematological change (Saslaw *et al.*, 1954). Dogs given CAP intramuscularly at 72 to 88 mg/kg over four weeks developed mild to moderate anaemia which recovered during the dosing period (Smith *et al.*, 1948). In dogs given oral CAP at 100 mg/kg for 3 months, a single animal showed a drop in erythrocyte count (Gruhzit *et al.*, 1949) whereas dogs given 250 mg CAP/kg orally for 4 weeks showed no haematological change (Radomski and Nelson, 1953). Anaemia and bone marrow changes were seen in dogs given oral doses of 250 mg CAP/kg for periods of up to 16 weeks, but weight loss and inappetance were also noted, findings which by themselves may cause bone marrow suppression (Reutner *et al.*, 1955). Cats given 50 mg CAP/kg/day intramuscularly for 21 d showed slight decreases in red cell count. The bone marrow showed erythroid hypoplasia with vacuolation, but again, severe inappetance was present (Penny *et al.*, 1967). At doses of up to 1500 mg CAP/kg/day, transient anaemia and reticulocytopenia were seen in orally-dosed ducks. Reticulocyte counts were maximally depressed 2 - 3 d after the start of dosing, but by 4 d after dosing, a rebound reticulocytosis was seen despite continued dosing (Rigdon *et al.*, 1954). This finding has been reproduced after 12 and 21 d in mice receiving 1250 and 2500 mg CAPS/d, respectively (Turton *et al.*, 2000). Firkin *et al.* (1974) reported a reduction in the reticulocyte counts of mice given subcutaneous (sc) CAP at 1800 mg/kg/d for 5 d, whereas Robin *et al.* (1981) noted no effect on peripheral blood counts of mice given 100 mg CAP/kg/d, 5 d/week, for 5 weeks.

Whilst CAP shows no ototoxicity when given systemically (Beaugard *et al.*, 1981), topical administration in guinea pigs caused damage to hair cells and to the organ of Corti (Proud *et al.*, 1968).

Embryotoxicity (increased rate of resorptions), decreased fetal growth and malformations were observed when CAP was administered to pregnant rats and mice at 2000 mg/kg/d, and rabbits at 1000 mg/kg/d (Fritz and Hess, 1971) and chick embryos (Blackwood, 1962)

1.4

Busulphan

1.4.1 Structure, Mode of Action, Therapeutic Indications

BU, 1,4-butanediol methanesulphonate, is a bifunctional alkylating agent with specific cytotoxicity for slowly proliferating or non-proliferating haemopoietic cells, especially of the myeloid lineage (Blackett and Millard, 1973).

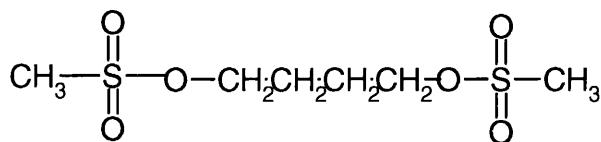


Figure 1.3: Chemical structure of BU

Alkylation of DNA and the formation of crosslinks prevents the separation of complementary DNA strands with the result that cell replication is prevented (Dunn, 1974; Hall and Tilby, 1992). The cytotoxicity of BU to proliferating granulocytes is the underlying property in its main therapeutic indication, that of reducing the total granulocyte mass in chronic myelogenous (granulocytic) leukaemia (CML) (Galton, 1953). Whilst not curative, BU is able to induce remission and relieve the symptoms of CML and improve the clinical state of the patient (Koeffler and Golde, 1981). However, recent trials have shown BU to be inferior to hydroxyurea (HU) for CML therapy. Median survival after HU is longer, and BU causes more frequent and serious complications, including irreversible cytopenias and pulmonary, hepatic and cardiac fibrosis. Alternatives to BU and HU therapy for CML now include IFN α and BMT. (Hehlmann *et al.*, 1993; Silver *et al.*, 1999). BU is also effective in related myeloproliferative states such as polycythaemia (PRV), essential thrombocythaemia and myelofibrosis. In the 40 or more years since its introduction, BU has also been employed against other malignancies, such as bronchial carcinoma, as a general immunosuppressant

after organ transplants, and in the treatment of autoimmune diseases, although BU's immunosuppressive properties are much less than that of other cytotoxic agents. More recently BU has been employed at high dosage alone, or in combination with irradiation or CY in preparatory regimes prior to bone marrow or haemopoietic stem cell transplantation (Buggia *et al.*, 1994).

For the treatment of CML in man, BU is administered at 0.06 mg/kg/day, with an initial dose of 4 mg until the total leucocyte count is reduced to $20 \times 10^9/L$, usually after 3 - 4 weeks (Koeffler and Golde, 1981). Treatment of PRV is 4 - 6 mg daily (EORTC, 1981) and of thrombocythaemia 2 - 4 mg daily. Plasma concentration of BU after oral dosing achieves only 5 - 6 % of the administered dose, and levels of iv administered BU are less than 10 % of total after 5 - 10 minutes (Bishop and Wassom, 1986). In myeloablative therapy prior to bone marrow transplantation, high doses of BU are administered, generally 1 mg/kg, four times daily for 4 d (Hassan *et al.*, 1991). The elimination half-life of BU is between 0.6 and 3 h after iv or ip injection (Hassan *et al.*, 1996). Although slightly higher than plasma levels of BU are achieved in a number of tissues, including spleen, bone marrow, liver, kidney and lung, selective uptake is not considered likely as an explanation for its specific effects against haemopoietic and other tissues (Bishop and Wassom, 1986). A water-soluble BU formulation, based on a dimethyl sulphoxide stock solution, has been described as yielding higher plasma concentrations in dogs than similar doses administered in tablet form (Ehninger *et al.*, 1995).

1.4.2 Toxicity in Man

Bone marrow depression, though recognised as a pharmacological property of BU, is nevertheless a dose-limiting toxic effect. Because BU is toxic to haemopoietic stem cells,

excessive or prolonged exposure to BU can lead to permanent aplasia (Weatherall *et al.*, 1969). Diffuse pulmonary fibrosis is a well-documented effect of standard BU therapy, occurring in up to 2.5 % of patients treated for CML. However, in high-dose regimens, as used for marrow transplant conditioning, sterility and gastrointestinal toxicity are commonly observed (Buggia *et al.*, 1994). Less frequent effects in man include jaundice, cataracts, adrenal insufficiency, endocardial fibrosis and epithelial alterations of the pancreas. Cytotoxic damage to the cervix, neurological, and gonadal tissues have also been recorded (Bishop and Wassom, 1986). The assignment of carcinogenic potential to BU is based on *in vitro* and animal testing (see Section 1.4.3, below) and the increased frequency of acute nonlymphocytic leukaemia and carcinoma development in patients treated with BU (Landaw, 1986; IARC, 1987).

1.4.3 Toxicity in Animals

In reproductive studies, BU has been shown to produce lasting effects on fertility, in male rats, mice, hamsters and other species with deletion of stem cells and spermatogonia, and in females by depletion of oocytes (Landaw, 1986). BU is teratogenic in mice, rats and rabbits, causing increased death rate of offspring, stunting, wasting and defects to the musculo-skeletal system. In both *in vitro* and *in vivo* systems, BU is genotoxic: mutagenicity tests for BU are positive with strain TA100 (Ames test), *Neurospora*, *Drosophila*, mouse lymphoma cells and human lymphocytes. In mice, BU induces dominant lethal mutations in oocytes and spermatozoa and chromosome aberrations in bone marrow cells, testes, oocytes and embryonic liver after ip dosing (Basler *et al.*, 1979). BU is positive in the mouse micronucleus assay (Landaw, 1986). Carcinogenicity studies with BU in laboratory species have been of short duration or incompletely reported. In studies to evaluate the development of pulmonary and mammary tumours in

rats and mice, no increased incidence was seen. However, the incidence of ovarian tumours and T cell lymphomas were increased after BU administration in mice (Upton, 1961; Conklin *et al.*, 1965; Bhoopalam *et al.*, 1986). The frequency of myeloid and other leukaemias and pulmonary tumours in mice was not increased by BU (Shimkin, 1954; Conklin *et al.*, 1965). BU administration causes cararacts in rats (Solomon *et al.*, 1955).

1.4.4 Haemotoxicity

Early studies in rats characterised the acute suppression and rebound of haemopoiesis (Elson, 1955). The reduced colony-forming ability of BU-treated rats demonstrated that BU had a specific inhibitory effect on CFU-S, primitive haemopoietic stem cells (Dunn and Elson, 1970), although committed granulocytic precursors (CFU-C, or CFU-GM) are also ablated by BU treatment (Delmonte, 1978). This effect is manifested by an abortive wave of erythropoiesis 3 - 5 d after a single dose in mice, as stem cell differentiation attempts to compensate for the early loss of lineage-committed precursors, the time lag reflecting the time to maturation (Boggs and Boggs, 1980). Stem cell injury in BU-treated mice has been further characterised by long-term marrow culture (Boyd *et al.*, 1986; Halka *et al.*, 1987). Depletion of murine stem cells is long-lasting after BU treatment (Morley *et al.*, 1975) and their ability to proliferate is reduced (Botnick *et al.*, 1979). In the face of such stem cell injury, committed granulocyte precursors (CFU-GM) and erythroid precursors (CFU-E) are unaffected in their response to stimulation of granulopoiesis (Fitchen and Cline, 1980) and erythropoiesis (Jelkmann and Bauer, 1980), respectively. BU also causes marrow stromal cell injury (McManus and Weiss, 1984; Molineux *et al.*, 1986) and damage to other tissues resulting from stem cell injury (Botnick *et al.*, 1979; Down *et al.*, 1989). Despite its highly myelosuppressive properties, BU is not markedly immunosuppressive (Santos and Tutschka, 1974; Yeager *et al.*,

1991), although lasting effects on lymphopoiesis are seen following BU treatment (Pugsley *et al.*, 1978; Andrews *et al.*, 1997).

1.5 The Investigations of AA Morley and the Development of a Mouse Model of Chronic Hypoplastic Marrow Failure

It was shown in 1974 by Morley and Blake (1974a) that ip administration of BU to female Swiss mice on four successive fortnightly occasions (20, 20, 20 and 10 mg BU/kg) induced persistent bone marrow hypocellularity (up to 240 d after the cessation of treatment). During this time, the majority of treated mice died of aplasia (Morley and Blake, 1974a). A proportion of BU-treated mice developed lymphoma. Animals appearing normal were sacrificed at intervals during the study. These animals were leucopenic, but with otherwise normal peripheral counts. Marrow cellularity was reduced on average to 58 % of controls, but CFU-S and CFU-C were reduced to 25 % and 42 % of control values respectively. These animals were termed “latent”, i.e., bearing residual marrow injury and a potential for marrow failure (Morley and Blake, 1974b). The stem cell lesion was confirmed by cross-transplantation experiments in BALB/c mice (Morley *et al.*, 1975) and “latent” mice were used in later experiments to demonstrate susceptibility to CAP (Morley *et al.*, 1976b; see below). In addition, BU induced a reduction of B and T lymphocytes of peripheral blood, bone marrow and spleen, and of thymic T lymphocytes (Pugsley *et al.*, 1978).

Morley and his colleagues also tested other cytotoxic drugs for their ability to produce residual marrow injury, and found similar properties for BCNU, mitomycin-C, melphalan and chlorambucil (Trainor and Morley, 1976; Trainor *et al.*, 1979).

BU not only reduced overall marrow cellularity, but also decreased transplantable pluripotent stem cells (CFU-S) and granulocytic progenitor cells (CFU-C or CFU-GM) (Morley and Blake, 1974b). When transplanted into BU-treated mice, normal bone marrow cells corrected the BU-induced lesion, suggesting that whilst stem cells were damaged, the marrow stroma, or microenvironment, was not (Morley *et al.*, 1975). However, deficiencies in stromal cell function were detected in long-term cultures from "latent" BU-treated mice (Hays *et al.*, 1982).

Morley *et al.* (1974a) had also demonstrated the acute *in vitro* depression of human and murine (Swiss mouse) granulocyte colony formation (CFU-GM) with CAP concentrations of 5 µg/mL and greater (mean concentration producing 50 % inhibition of CFU-GM in man, 14.3 µg/mL, and in mice, between 1 and 10 µg/mL).

Early studies in rabbits had shown that BU (10 mg/kg orally, weekly) and CAP (300 mg/kg/d, orally), when administered together for four weeks, produced a greater depression on circulating and bone marrow granulocytes and on total marrow DNA content than either drug administered separately (Lu *et al.*, 1956/7). Similarly, in rabbits given BU (10 mg/kg/d, sc) and CAP (1 g/kg/d, sc) leucocytes, platelets, but not reticulocytes were depressed after two weeks to a greater extent than in animals given either BU or CAP alone (Speck and Moeschlin, 1968).

Morley (Morley and Blake, 1974a; 1974b) considered that residual marrow damage in man, undetectable by standard haematological tests on peripheral blood, might predispose susceptible individuals to CAP-induced AA. Morley and his co-workers therefore carried out a study where CAP was administered to female BALB/c mice previously treated with

BU. BU was administered ip fortnightly at 20, 20, 20 and 10 mg/kg over a six week period. After a further eight weeks, to allow for the acute toxicity of BU to resolve, CAPS was given *ad libitum* in the drinking water at 5 mg/mL, such that serum concentrations of CAP were between 2.5 and 4.0 µg/mL. At various timepoints up to 150 d after the introduction of CAPS, reductions in tibial nucleated cells, CFU-S and CFU-GM were seen in the BU/CAPS-treated animals, whereas CAPS-only treated animals did not differ from controls for these parameters (Morley *et al.*, 1976b).

The BU-induced residual injury to murine marrow has been shown to be successful in predicting agranulocytosis in man. Captopril, an angiotensin I-converting enzyme inhibitor, induces agranulocytosis in some patients, especially those with renal impairment, or in those being treated concurrently with other potentially myelotoxic drugs (Erslev *et al.*, 1982). Preclinical rodent studies had shown no effect of captopril on neutrophil counts, although there were occasional cytopenias in dogs (Boyd *et al.*, 1982); Using the BU regimen described by Morley and Blake (1974a), Boyd *et al.* (1982) demonstrated additive suppression of CFU-GM growth in Swiss mice also given high-dose captopril. The effect was persistent, being still present one year after the initial BU dose (26 weeks after the start of captopril administration; Boyd *et al.*, 1982).

Standard preclinical drug testing in animals is not able to detect idiosyncratic AA (Vincent, 1984; Utrecht, 1992). Thus a model of chronic hypoplastic marrow failure in the mouse which was capable of predicting idiosyncratic toxicity (AA) in man would be of considerable importance. Substitution of novel compounds in the place of CAPS in Morley's BU mouse model could generate preclinical safety assessment data which would permit greater assurance of the likelihood of adverse events in man. However, a

review of the literature carried out at the beginning of the present investigations revealed that the model as described by Morley had never been fully validated either with CAP or with other drugs or chemicals associated with AA. Indeed, many authors have stated that animal models of drug-induced AA do not exist (Saslaw *et al.*, 1954; Yunis and Bloomberg, 1964; Wallerstein *et al.*, 1969; Benestad, 1979; Appelbaum and Fefer; 1981; Vincent, 1986; Chaplin, 1986b; Holt *et al.*, 1993; Nakao, 1997; Young and Maciejewski, 1997).

1.6 Objectives of the Present Work

The primary objective of the present investigations was therefore to develop a model of BU-induced chronic hypoplastic marrow failure in the mouse. The B6C3F₁ strain would be employed, as this strain was, at the time, widely used for toxicity testing, and considerable in-house knowledge existed. The methods employed for the analysis of blood and haemopoietic tissue would be those available in the routine toxicological haematology laboratory, calibrated specifically for murine blood. These methods would offer greater sensitivity than those available to earlier workers and provide a simple and rapid characterisation of the state of hypoplastic marrow failure.

BU-treated mice would then be given CAP, a drug which has been strongly associated with AA in man, the purpose being to determine whether CAP-induced Type II/B (irreversible) haemotoxicity could be demonstrated. Initial work would be aimed at determining maximum tolerated dose levels for BU and CAPS in the female B6C3F₁ mouse, and in characterising the magnitude and time course of BU- and CAPS- induced haemotoxicity. Thereafter, B6C3F₁ mice would be treated with BU and CAPS. This would allow the characterisation of any late haemotoxicity due to BU administration, and if successful, demonstrate additional toxicity caused by CAPS. Successful validation would then permit the use of the model with BU in conjunction with other compounds known to induce AA in man (eg phenylbutazone, or other NSAIDs, gold salts). Thereafter, the model could be employed in conjunction with new drugs during development, as part of a safety testing programme, thereby providing an assessment of the potential of a new drug to cause haemotoxicity in man.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Female B6C3F₁ mice (Small Animal Breeding Unit, Glaxo Wellcome Research and Development, Ware, Herts, or Charles River, Margate, Kent, or Harlan Olac, Bicester, Oxon), 12 - 20 weeks old, were housed in groups of 5 or 6, bedded on wood shavings, and given diet (Rat and Mouse No. 1, SDS Ltd., Witham, Essex) and drinking water *ad libitum*. A temperature of 22°C ± 1°C was maintained, with a relative humidity of 45 - 70 % and a 12:12 h light:dark cycle (lights on at 06.00 h). Animals were acclimatised for at least 5 d before the start of each experiment and were observed daily for signs of ill-health, but more frequently after dosing. Animals were randomly assigned to dose groups using computer-generated tables.

2.2 Dosing

2.2.1 Busulphan

BU (Sigma Chemical Co. Ltd., Poole, Dorset) was dissolved in acetone at a concentration of 16.7 mg/mL. The BU solution was administered by ip injection after dilution with distilled water (Water for irrigation, BP) to give a constant dose volume of 10 mL/kg body weight. Control mice were given acetone with distilled water at the same dose volume.

2.2.2 Chloramphenicol Succinate

For administration in drinking water, 100 mg/mL stock solutions of CAPS (Sigma) prepared in distilled water (Water for irrigation, BP) were further diluted in tap water and placed in darkened glass water bottles which were replaced twice weekly with freshly

prepared dosing solutions. Water bottles were weighed before and after each dosing period to determine the volume consumed. For iv dosing, mice were given CAPS dissolved in distilled water (Water for irrigation, BP) at a rate of 10 mL/kg body weight. For gavage dosing, mice were given CAPS in Water for irrigation BP at a constant rate of 10 mL/kg.

2.3 Haematological Measurements

2.3.1 Blood Samples

Animals were killed by CO₂ overdose and 0.5 mL blood samples for cell counting were taken from the posterior vena cava and anticoagulated with 1.5 mg dipotassium EDTA/mL blood (Teklab, Sacriston, Durham). For the determination of haemolytic potential, murine blood, taken as above, and blood from healthy human volunteers, taken by antecubital venepuncture, were anticoagulated with heparin at 15 - 20 U/mL (Teklab, Sacriston, Durham).

2.3.2 Peripheral Blood: Full Blood Count, Differential Leucocyte Count and Reticulocyte Count

Full blood counts and differential leucocyte counts were obtained with a Bayer H*1 haematology analyser (Bayer Ltd., Newbury, Berks) (Ross and Bentley, 1986). Optimal separation of platelets and erythrocytes in animal blood is achieved in this system by differentiation of signals generated by scatter of laser light (Zelmanovic *et al.*, 1992; Byrne *et al.*, 1994). The percentages of microcytic and macrocytic erythrocytes were obtained, after isovolumetric spherling and partial fixation, from the forward light scatter signals exceeding cell volume thresholds for the mouse (microcytes <25 fL; macrocytes >75fL) and of hypochromic erythrocytes from the side scatter signals for cells <18 g

Hb/dL (Tycko *et al.*, 1985). Leucocytes, excluding basophils, were differentiated by cluster analysis of peroxidase content and volume, measured by absorption of right-angle scatter and forward scatter of tungsten light respectively, against an archetype for leucocytes of the appropriate species (Davies and Fisher, 1991). An estimate of the myeloperoxidase content of neutrophils (MPXI) was generated simultaneously. This index of enzyme activity is expressed in arbitrary units spanning zero, the approximate mean value for man. Basophils were counted in a separate channel as phthalic acid-resistant intact cells; other cells in this channel were detected as stripped nuclei. The basophil channel also generates a measure of nuclear lobularity (lobularity index) in neutrophils and eosinophils compared to mononuclear cells, but owing to the relatively high proportion of lymphocytes in rodent blood, lobularity index is not valid and is not reported here. The H*1, with veterinary software (Versions 1.0 - 3.0; Bayer Ltd, Swords, Dublin), was validated in-house for use with dog, rat and mouse blood (Andrews and Mifsud, 1990; McKinnon, 1994)

Reticulocytes were counted using a Sysmex R-1000 reticulocyte analyser (Sysmex UK Ltd, Milton Keynes, Bucks; Tichelli *et al.*, 1990) with photomultiplier voltage gain adjusted optimally for mouse blood (Fuchs and Eder, 1991; Takami and Sakata, 1991). Reticulocytes were differentiated from mature erythrocytes by fluorescence after staining with auramine O. Reticulocyte maturity was assessed according to fluorescence intensity which is subdivided into three fractions: high, mid and low fluorescence ratio (HFR, MFR, LFR). In-house validation of the R-1000 has been performed for dog, rat, mouse and marmoset blood (Hickson, 1992).

2.3.3 Bone Marrow

Bone marrow cellularity was assessed by flushing the contents of one femur into 10mL of phosphate buffered saline, and, after thorough mixing, obtaining the nucleated cell count from the basophil (leucocyte) channel of the H*1. Where the presence of fat particles was evident in the analyser cytogram, correction of the count was performed (Bentley *et al.*, 1995). Smears made from the tibia or opposite femur were stained with May-Grünwald-Giemsa stain and examined microscopically for morphological assessment and enumeration of myeloid, erythroid, lymphoid and other cells in 200 cell differential counts.

2.3.4 Spleen

Nucleated cell counts of spleen suspensions were obtained using the basophil channel of the H*1. Suspensions were prepared by scissor-mincing after removal of capsular material and dispersion by cavitation with a 50 mL syringe. Spleen imprints were made by touching the cut surface of the organ on a microscope slide which was subsequently stained with May-Grünwald-Giemsa stain. Where histological processing was also carried out, spleens were first weighed and cut into two roughly equal portions. Cell counts were performed on one weighed portion and cellularity of the whole spleen extrapolated from the two weights.

2.4 Flow Cytometry

2.4.1 Lymphocyte Subset Analysis

Lymphocyte subset analysis was performed using two different methods (Andrews *et al.*, 1997) owing to the availability of instrumentation and reagents at the different times when the analyses were carried out.

2.4.1.1 Method 1

0.5 ml blood, anticoagulated in 0.106M trisodium citrate (9 vol blood:1 vol citrate) was added to 3.0 mL ammonium chloride for erythrocyte lysis and centrifuged at 350g for 10 min at 4°C. The pellet was then washed in RPMI 1640 culture medium + HEPES buffer with 5 % fetal calf serum and 0.05 % azide, and centrifuged at 350g for 5 min at 4°C . The resuspended pellet was subdivided into three aliquots which were treated with Caltag antibodies (Bradsure Biologicals Ltd., Loughborough, Leics) as follows: (1) rat anti-mouse L3/T4 (CD4)-tricolor (2µL per 50 µL cell suspension) and rat anti-mouse Ly2 (CD8a)-fluorescein isothiocyanate (FITC) (5 µL per 50 µL cell suspension); (2) goat anti-mouse IgG (H+L)-FITC (2.5 µL per 100 µL cell suspension); (3) RPMI 1640 only, for autofluorescence control. After incubation for 30 min at 4°C, three further washes were performed as before. Samples were finally reconstituted in RPMI 1640 for two-colour analysis using a Coulter EPICS flow cytometer (Coulter Electronics, Luton, Beds).

2.4.1.2 Method 2

0.5mL EDTA blood was treated with RPMI 1640 + HEPES buffer as before, but the washed pellet was treated with distilled water to lyse the erythrocytes. After antibody concentration had been optimised by titration, three colour analysis of Pan T, CD4 and CD8 cells, and two colour analysis of Pan T and B cells were performed simultaneously using Caltag antibodies (Bradsure Biologicals) and a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Cowley, Oxon). Three colour analysis was with mouse-anti mouse Thy1.2 (Pan T)-phycoerythrin (PE), rat-anti mouse L3/T4 (CD4)-tricolor and rat-anti mouse Ly2 (CD8a)-FITC. Two-colour analysis was with mouse-anti mouse Thy1.2 (Pan T)-PE and goat-anti mouse IgG (H+L) (B cells)-FITC. Antibody concentrations used were: anti-Thy 1.2: 5 µL in 50 µL; anti-IgG: 2 µL in 50 µL; anti-L3/T4: 2 µL in 50 µL;

anti-Ly2: 5 µL in 50 µL; incubations were for 60 min. Autofluorescence of normal samples was 0 % in all cases. Isotype controls gave the following background levels of fluorescence: rat IgG 2b-FITC (for Ly2), 1.8 - 2.5 %; rat IgG 2b-Tricolor (for L3/T4), 6.0 - 6.4 %; mouse IgG 2b-PE (for Thy 1.2), 0 %.

2.4.2 Bone Marrow Stem Cell Analysis

To tubes containing 100 µL bone marrow suspension in phosphate buffered saline were added 5 µL rat anti-mouse CD117 (Serotec, Oxford) and 10 µL F(ab') rabbit anti-rat-FITC (Serotec). Isotype controls were prepared in the same way, but with mouse isotype control (Serotec) substituted for the CD117 antibody. Samples were analysed using a Becton Dickinson FACScan with CellQuest software (Becton Dickinson)

2.5 Bone Marrow Progenitor Cell Assays

Femoral bone marrow was flushed into 1 mL Iscove's Modified Dulbecco's Medium (Life Technologies, Paisley, Scotland) under sterile conditions. 0.3 mL of this cell suspension was resuspended in 3 mL Methocult GF M3434 (Metachem Diagnostics, Northampton) culture medium, containing 0.9 % methylcellulose in Iscove's Modified Dulbecco's Medium, 15 % fetal calf serum, 10⁻⁴ M 2-mercaptoethanol, 2nM L-glutamine, 1% bovine serum albumin, 10 µg/mL bovine pancreatic insulin, 200 µg/mL human transferrin, 10 ng/mL recombinant murine (rm) IL-3, 10 ng/mL recombinant human IL-6, 50 ng/mL rm SCF and 3U/mL rm EPO. The tubes were vortexed and allowed to stand for 5 min at 37°C in a water bath before being dispensed into cell culture multi-well plates. The plates were incubated at 37°C in a CO₂ incubator. BFU-E were counted after 10 d incubation, and CFU-GM and CFU-E after 12 d, on the same plates. The colonies were identified morphologically and counted using an inverted microscope. BFU-E appeared as three to

eight clusters per colony of up to 100 cells. The clusters were circular in shape and contained some haemoglobin, giving the colony a distinctive red hue. CFU-E were tight clusters of cells containing 10 - 60 cells per colony and appeared more deeply pigmented. CFU-GM colonies resembled a single large star-shaped cluster of approximately 25 cells, which were devoid of colouration. Each sample was assayed in triplicate.

2.6 Histopathology

Autopsies were carried out on animals killed by CO₂ overdose. Tissues (sternum, spleen, thymus, mesenteric and cervical lymph nodes, liver and lungs) were removed and placed in 10 % buffered formalin. Eyes were removed and fixed in Bouin's fixative. Sternebrae were decalcified for 3 d in Kristenson's solution. Tissues were embedded in paraffin following fixation, 3 - 4 µm sections prepared and stained with haematoxylin and eosin. Some tissues were stained by Perls' Prussian blue technique and counterstained with eosin or Martius Scarlet Green.

2.7 Chloramphenicol and Chloramphenicol Succinate Determination

2.7.1 Method 1

CAP was assayed using the Emit CAP Assay Kit (Syva Company, Maidenhead, Berks), a competitive immunoassay system using G6PDH labelled CAP. Levels of reduced nicotinamide-adenine-dinucleotide (NADH) generated by residual G6PDH were measured on a Cobas Mira centrifugal analyser (Roche Diagnostics, Welwyn, Herts). Subsequently, stock CAPS solution (100mg/mL) and dosing solution (CAPS in drinking water, 4.0 mg/mL) were analysed daily to assess stability of CAP and CAPS for 10 d.

2.7.2 Method 2

CAP and CAPS were determined using high performance liquid chromatography (HPLC)

on a phenyl-bonded silica column and detection effected by ultraviolet spectrophotometry at 275nm using a Shimadzu SPD-6A UV detector (Holt *et al.*, 1990, 1995). Bone marrow samples for CAP and CAPS determination were prepared by flushing the contents of one femur into isotonic saline, centrifuging the suspension at 1200g for 10 minutes, discarding the supernatant then freezing the pellet at -20°C. Portions of liver (approximately 5g) for CAP and CAPS analysis were removed and immediately frozen by plunging into dry ice.

2.8 Osmolality

Osmolality of iv CAPS dosing solutions, determined by depression of freezing point, was performed using a Fiske 2400 osmometer (Vitech, Horsham, Sussex).

2.9 *In vitro* Haemolysis Tesing

The *in vitro* haemolytic potential of solutions was measured after incubation with equal volumes of mouse and human blood at 37°C for 45 min, based on the method of Prieur *et al.* (1973). Negative (0 % haemolysis) and positive (100 % haemolysis) controls were prepared using isotonic saline and 1 % aqueous saponin solution, respectively. Hb was measured in the supernatant after centrifugation against a standard curve at 560, 576 and 592 nm (Cripps, 1968) using a Shimadzu UV-160 spectrophotometer (Heraeus, UK).

2.10 Statistical Analysis

Student's t test was used to compare treated and control groups at the same timepoint, using RS/1 software (BBN, Cambridge, Mass.) or Microsoft Excel. In Experiment 6, four separate analyses of haematological data, including pairwise group analysis, were

performed, using Student's t test. These comparisons are described in Chapter 5, Results.

For the analysis of intercurrent deaths (ICD) in Chapter 5, Cox's Proportional Hazards

Model (Cox and Oakes, 1984) was used.

CHAPTER 3: DOSE RANGING STUDIES WITH BUSULPHAN: CHARACTERISATION OF SHORT-TERM MYELOTOXICITY

3.1 Introduction

The literature suggests that a range of BU doses from 5 to 100 mg/kg, when administered ip, either as a single dose or as repeated doses, can cause myelotoxicity in a wide range of mouse strains (Freireich *et al.*, 1966; Morley and Blake, 1974a; Boggs and Boggs, 1980; Fitchen and Cline, 1980; Jelkman and Bauer, 1980; Anderson *et al.*, 1982). However, there appear to be no reports of BU-induced blood changes in the B6C3F₁ mouse, a strain which has been used extensively for toxicity testing. The LD₅₀ for a single ip BU dose in the mouse has variously been given as 30 mg/kg for female Swiss mice (Morley and Blake, 1974a), 40 mg/kg for female Rf/Up and (101 x C3H)F₁/Cum mice (Asano *et al.*, 1963), between 60 and 90 mg/kg for male CBA mice (Alexander and Connell, 1960) and 125 mg/kg (sex and strain not reported; Sternberg *et al.*, 1958). In the model of chronic hypoplastic marrow failure (Morley and Blake, 1974a), Swiss mice and BALB/c mice received cumulative doses of 70 mg/kg (four doses of 20, 20, 20 and 10 mg/kg at intervals of 14 days).

For the initial experiment in the present study with the B6C3F₁ mouse (Experiment 1) an initial range of 10-60 mg BU/kg, with the drug administered as a single dose with increments of 5 mg/kg, was selected. Haematology measurements were to be performed at two weeks after dosing to assess myelotoxicity. This would establish a maximum tolerated single dose (MTSD) of BU. A range of dose levels would then be chosen (Experiment 2) with the highest level somewhat lower than the MTSD. These doses would then be administered successively, on four occasions, each dose separated by two

weeks, following the regimen used by Morley and Blake (1974a). Haematology measurements would be performed at two weeks after dosing to compare the myelotoxic effects of single (Experiment 1) and repeat dosing (Experiment 2). However, samples would also be studied in Experiment 2 at six weeks post dosing, to assess whether the blood changes had resolved.

An examination of blood values at six weeks post dosing in Experiment 2 was planned as it was intended that a period of time should elapse before beginning the administration of CAPS. Whilst early work with rabbits examined the effects of BU and CAP when co-administered (Lu *et al.*, 1956/57; Speck and Moeschlin, 1968), Morley *et al.* (1976b) allowed six weeks to elapse after BU treatment before beginning the administration of CAPS, so that early BU toxicity could resolve. Periods of between two weeks (Pazdernik and Corbett, 1980) and twenty weeks (Robin *et al.*, 1981) between the dosing of BU and the beginning of CAP or CAPS administration have been allowed by various workers. However, it was considered that if evidence of myelosuppression persisted at the six-week timepoint in Experiment 2, but the clinical condition of the animals appeared normal, this point in time could be used as the starting point for CAP dosing in later experiments.

Experiment 3 was designed to measure more closely the extent of myelosuppression and its recovery after administering the MTSD of BU. Haematological measurements would be performed at intervals over a period of ten days after dosing, to examine the short-term haemopoietic response to BU during the two-week interval between repeat doses (Experiment 2).

In many early reports on the myelotoxicity of BU, rather limited haematological techniques had been employed. For example, Elson (1955) performed only haemoglobin estimation and leucocyte count on manual dilutions of blood from the tail veins of rats, and calculated the absolute number of neutrophils and lymphocytes from the percentage of these cells in blood smears. Similarly, Morley and Blake (1974a) describe the use of "standard techniques" for peripheral cell counts, with leucocyte, platelet, tibial cell and reticulocyte counts being performed visually. Early attempts at automated haematological analysis of non-human blood had often been hampered by the limited volume of blood available from small animals, or by the small erythrocyte volume, platelet interference, and other individual cellular characteristics (Zelmanovic *et al.*, 1992; Byrne *et al.*, 1994). However, at the time the present project commenced, opportunities had become available to perform detailed analysis of data generated by analytical methodology specifically programmed for rodent blood (Davies and Fisher, 1991; Fuchs and Eder, 1991), therefore these techniques were used in the present investigation.

The aims of these first three experiments were therefore to determine the MTSD of BU in the female B6C3F₁ mouse (Experiment 1); to determine the maximum tolerated repeat dose of BU using the dosing protocol described by Morley *et al.* (1974a) (Experiment 2); and to characterise the BU-induced haematological lesion and the time course of its development after a single dose (Experiment 3) using dedicated automated flow cytometric analysers.

3.2 Materials and Methods

Mice were obtained from the Small Animal Breeding Unit, Glaxo Wellcome Research and Development, Ware, Herts. BU dosing, blood, bone marrow and spleen sampling and analysis were as described in Chapter 2.

3.2.1 Experiment 1: Busulphan Dose Ranging: Single Dose Study with Sampling at 14 Days

Single doses of BU were administered ip at 0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mg/kg to four mice at each dose level. Controls received ip acetone in water (vehicle) only. After the administration of BU, animals were observed frequently over a period of four hours to study tolerability. Animals receiving BU dosages that were well tolerated were weighed and observed daily for 14 d. At 14 d post dosing, blood samples were taken for full blood count, differential leucocyte count and reticulocyte count.

3.2.2 Experiment 2: Busulphan Dose Ranging: Repeat Dose Study with Sampling at 14 and 42 Days

Groups of twelve mice were given four ip doses of BU at fortnightly intervals at 0, 10, 15, 20, 25, 30, 35 and 40 mg/kg. Blood samples were taken for a full blood count, differential leucocyte count and reticulocyte count from six mice in each dose group after 14 and 42 d. Body weights were recorded twice weekly.

3.2.3 Experiment 3: Acute Toxicity of Busulphan: 10-day Study with Sequential Sampling Following a Single Dose

Three groups of 30 mice were given a single ip dose of BU at 0, 35 or 45 mg/kg. At 1, 2, 3, 4, 7 and 10 d post dosing, five mice from each group were killed and blood samples taken for full blood count, differential leucocyte count and reticulocyte count. Bone marrow and spleen cellularity was determined and marrow smears were prepared for morphological examination and differential cell counts. Body weights were recorded daily.

3.3 Results

3.3.1 Experiment 1: Busulphan Dose Ranging: Single Dose Study with Sampling at 14 Days

3.3.1.1 Clinical Observations

BU was well tolerated at levels up to 45 mg/kg. Transient signs of toxicity including unsteadiness of gait, lack of coordination and subdued demeanour were noted immediately after dosing, at 35, 40 and 45 mg BU/kg, but at these dose levels the animals subsequently appeared well. There was no significant effect or trend apparent on body weights in animals dosed at up to 45 mg/kg over the following 14 d. However, due to toxicity, the majority of animals receiving 50, 55 and 60 mg BU/kg died or were killed *in extremis* in the 24 h following drug administration, and the remainder in these groups died or were killed *in extremis* in the ensuing 72 h.

3.3.1.2 Haematological Measurements

Erythrocyte count, haemoglobin and haematocrit were not significantly affected by BU administration (Fig. 3.1) at 14 d post dosing although a reduction in the group mean for these parameters was evident at 45 mg/kg due to one animal in the group of four showing moderate anaemia. The percentage and absolute reticulocyte counts were unaffected, but significant increases in the percentage of macrocytic erythrocytes were seen at all BU dose levels. MCV was significantly decreased at BU dose levels of 10 to 25 mg/kg and significantly increased at 40 and 45 mg BU/kg. Platelet counts showed significant reductions at all dose levels (Fig. 3.2), with a clear relationship with the dose administered. Platelet counts in animals receiving 45 mg/kg were reduced to 21 % of the control mean. Lymphocyte counts showed moderate elevations 14 d after a single BU

dose of 10 to 25 mg/kg, but at 30 to 45 mg/kg, counts were not different from the control value (Fig. 3.3). The total leucocyte count showed a similar pattern, reflecting the proportional composition of lymphocytes. The neutrophil count was significantly elevated at 15 mg BU/kg but significantly depressed at 40 and 45 mg/kg (Fig. 3.4). Neutrophil myeloperoxidase index (MPXI) was unaffected. There was a dose-related trend for the depression of monocyte, eosinophil and LUC counts at levels of 25 mg/kg and above (Fig. 3.5), although eosinophils were moderately reduced at 10 and 15 mg BU/kg. Basophil counts were less noticeably affected.

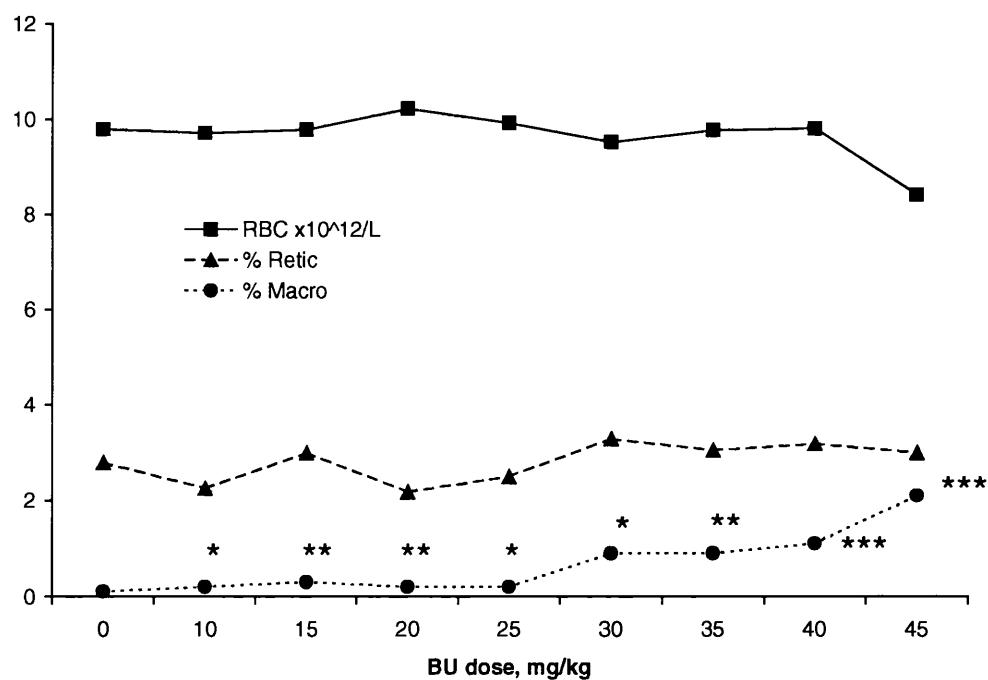


Figure 3.1: Mean erythrocyte count, percentage reticulocytes and macrocytic cells at 14 d after a single BU dose. Points denoting each measurement are linked for clarity. n=4; *significantly different to control value, p<0.05; **p<0.01; *p<0.001.,**

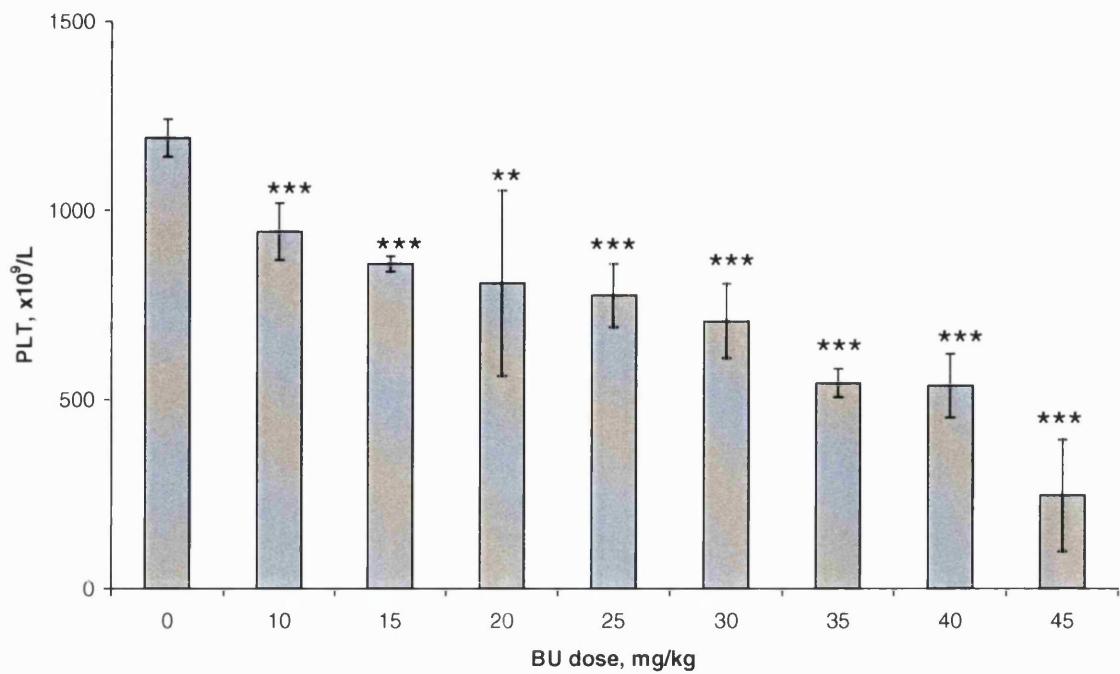


Figure 3.2: Mean and sd of platelet counts at 14 d after a single BU dose. n=4; ** significantly different to control value, p<0.01; * p<0.001.**

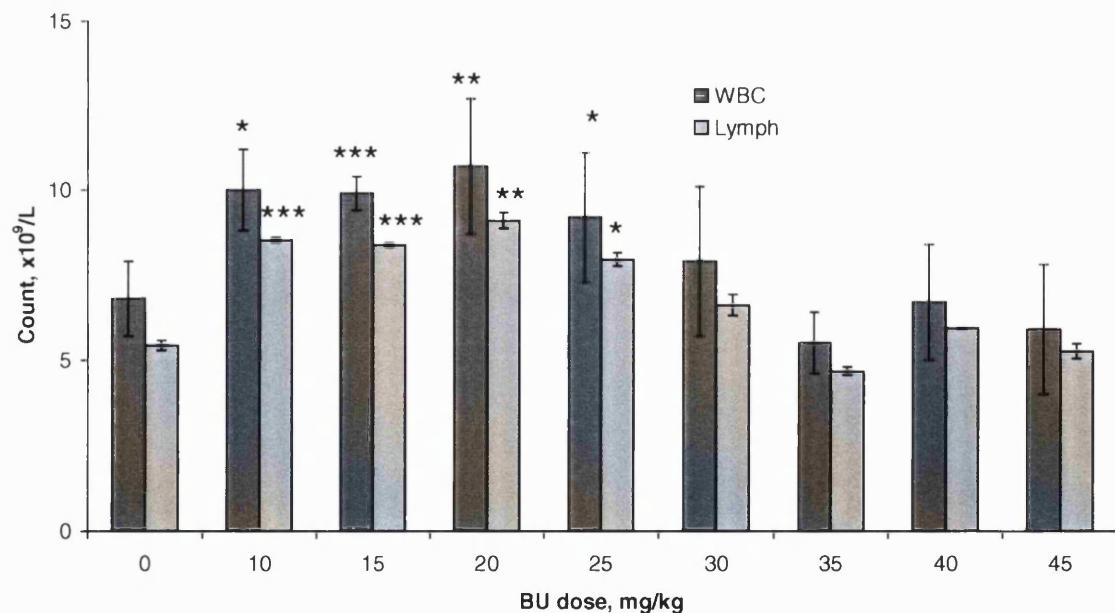


Figure 3.3: Mean and sd of total leucocyte and lymphocyte counts at 14 d after a single BU dose. n=4; * significantly different to control value, p<0.05; ** p<0.01; * p<0.001.**

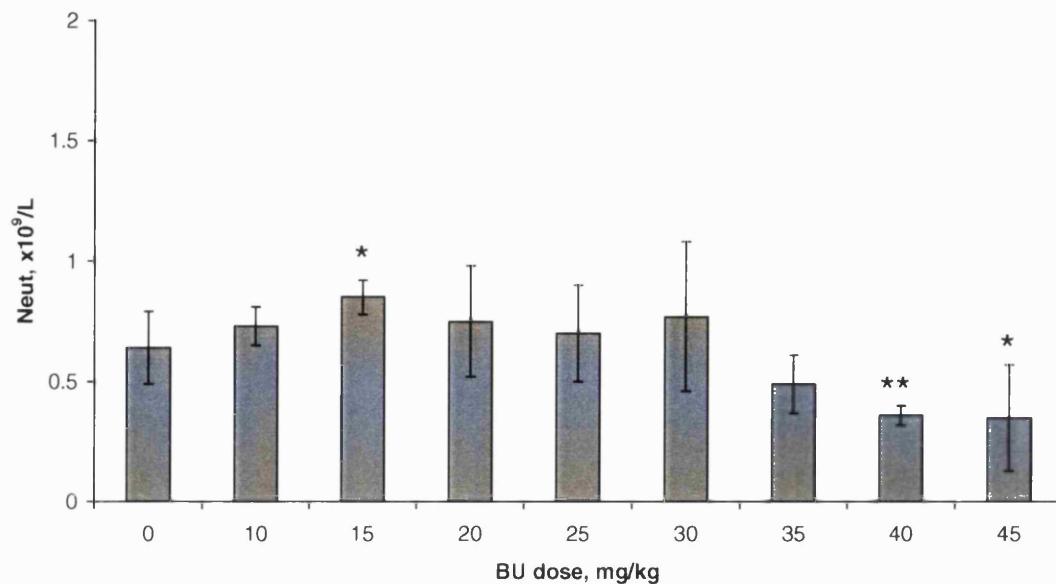


Figure 3.4: Mean and sd of neutrophil counts at 14 d after a single BU dose. n=4; * significantly different to control value, p<0.05; ** p<0.01.

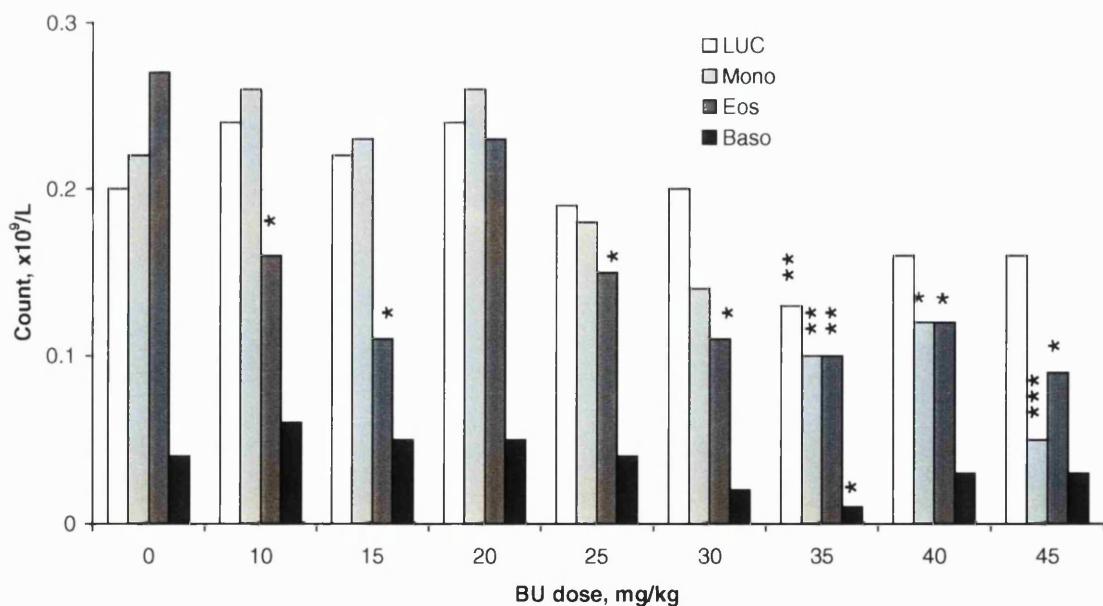


Figure 3.5: Mean leucocyte counts, other than neutrophils and lymphocytes, at 14 d after a single BU/kg. At 45 mg BU/kg, counts are depressed as follows: LUCs, 75 % of control value; monocytes, 22 %; eosinophils, 33 %; basophils, 80 %. n=4; * significantly different to control value, p<0.05; ** p<0.01; * p<0.001. Asterisks are aligned vertically for clarity.**

3.3.2 Experiment 2: Busulphan Dose Ranging: Repeat Dose Study with Sampling at 14 and 42 Days

3.3.2.1 Clinical Signs

No adverse clinical events, other than the transient signs seen immediately after dosing and described earlier (Experiment 1), were evident in repeat-dosed animals. Body weight gain did not differ significantly in BU dosed animals as compared to the controls during the dosing period, or over the following 42 d. There were no mortalities.

3.3.2.2 Haematology

Significant reductions in erythrocyte counts were seen at 30 mg/kg BU and above at 14 and 42 d (Fig. 3.6). An upward trend in reticulocyte counts at increasing BU dose levels was seen at 14 d only, but the increases were not significant. However, dose-related increases in the percentage of macrocytic cells were evident, particularly at 14 d, and some of these increases were statistically significant, especially at 30 mg BU/kg and above. The magnitude of the change in all three parameters was similar at both timepoints. MCV was significantly increased at all dose levels at 14 d post dose, showing a dose-related effect, but at 42 d, this effect was only present at 30 to 40 mg BU/kg.

Significant reductions in platelet count were seen at all dose levels at both 14 and 42 d post dosing (Fig. 3.7), and a dose-related trend was present at both occasions. The reduction in platelet count (to 39 % of control value) seen in animals receiving 40 mg BU/kg at 42 d was less than the reduction at 14 d (to 17 % of control value), however the individual counts at d 42 showed greater variability than at d 14.

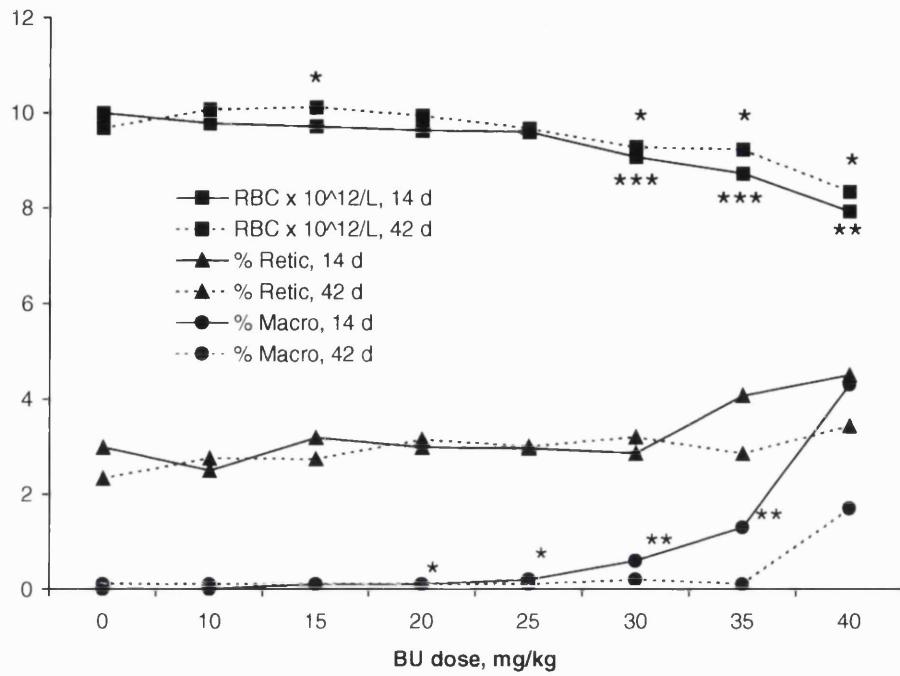


Figure 3.6: Mean erythrocyte counts and reticulocyte and macrocyte percentages at 14 and 42 d after four fortnightly BU doses. Measurements are linked for clarity. n=6; * significantly different to control value, p<0.05; ** p<0.01; *** p<0.001.

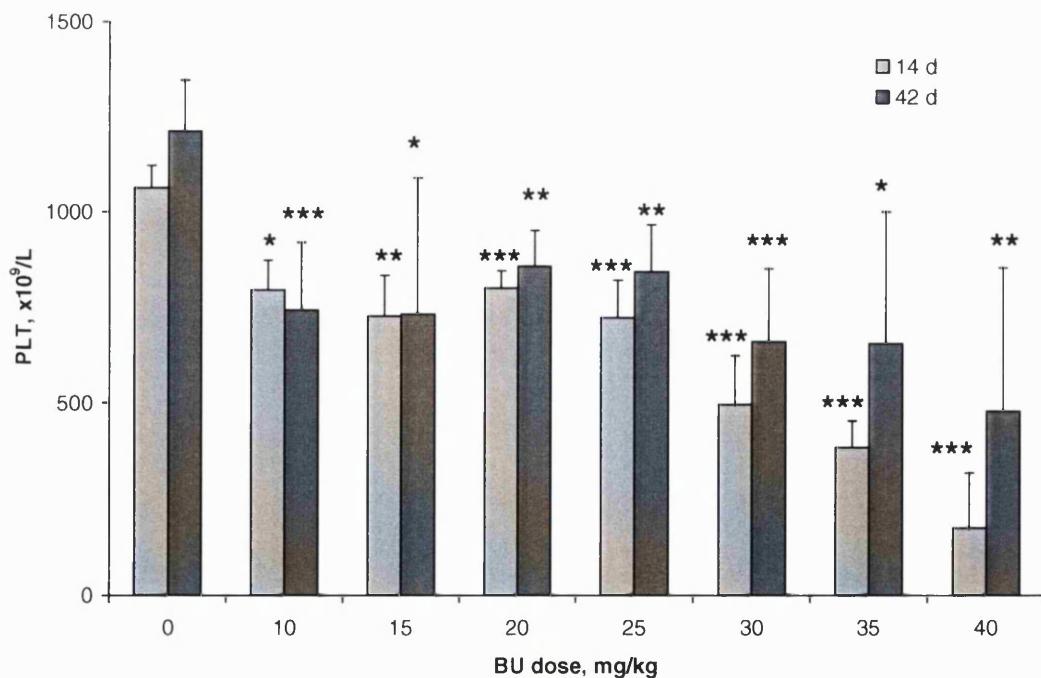


Figure 3.7: Mean and sd of platelet counts at 14 and 42 d after four fortnightly BU doses. n=6; * significantly different to control value, p<0.05; ** p<0.01; *** p<0.001.

Lymphocyte counts were elevated after 14 d at 10 to 25 mg BU/kg, but were reduced at 30 to 40 mg/kg (Fig. 3.8) but none of these alterations achieved significance. After 42 d, lymphocyte counts at 10 to 25 mg/kg were similar to controls but were significantly reduced at 30 to 40 mg/kg. There was a trend for reduction in neutrophil count at 25 to 40 mg BU/kg at 14 d and at 35 and 40 mg/kg at 42 d, but these reductions were not significant (Fig. 3.9). Increases in MPXI were present at both timepoints at 30 to 40 mg BU/kg, but again the increases were not statistically significant. Significant dose-related reductions of other leucocyte counts were also present at 14 d after dosing; in animals receiving 40 mg BU/kg the monocyte count was 24 % of the control value, LUC count 42 %, eosinophils 36 % and basophils 60 %. Forty-two d after dosing, counts for these cell types were less markedly reduced: monocytes were 59 % of the control value, LUC 57 % and basophils 67 %, but these reductions were nevertheless statistically significant ($p<0.05$). The eosinophil count was unaffected at d 42.

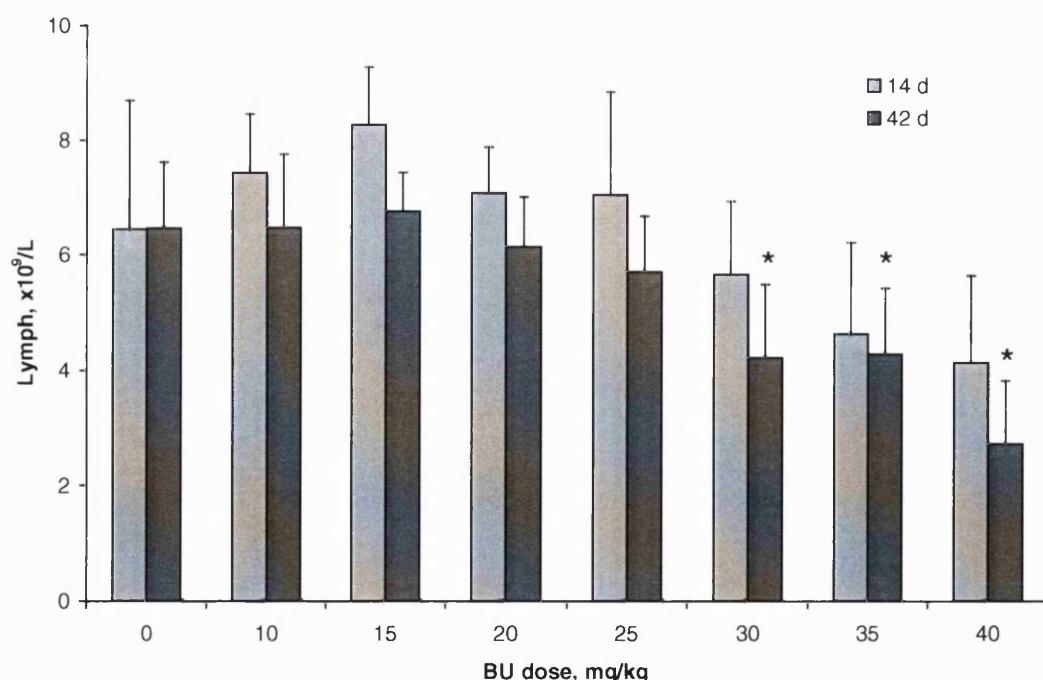


Figure 3.8: Mean and sd of lymphocyte counts at 14 and 42 d after four fortnightly BU doses. n=6; *significantly different to control value, $p<0.05$.

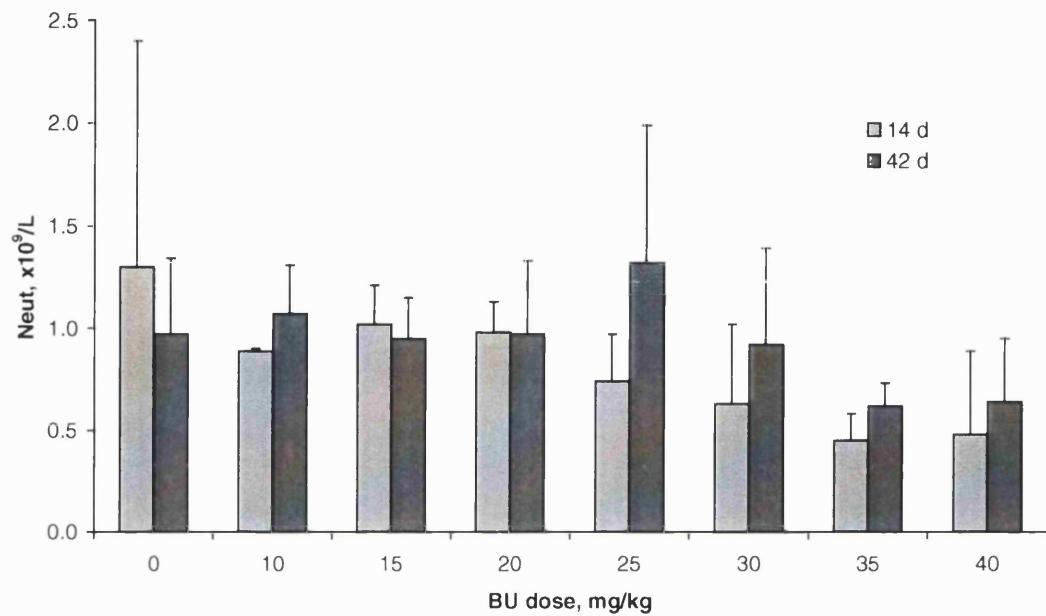


Figure 3.9: Mean and sd of neutrophil counts at 14 and 42 d after four fortnightly BU doses. n=6.

3.3.3 Experiment 3: Acute Toxicity of Busulphan: 10-day Study with Sequential Sampling Following a Single Dose

3.3.3.1 Clinical Signs

Animals appeared well at all times.

3.3.3.2 Haematology

The erythrocyte count was significantly depressed at 3 d after dosing at 45 mg BU/kg and remained so until d 10 post dose, although by this time there was evidence of recovery (Fig. 3.10). At 35 mg/kg, there was no significant depression in erythrocyte count. The reticulocyte count was depressed significantly 2 d after dosing at 45 mg/kg (but not at 35 mg/kg), but by d 7 had returned to a value slightly greater than the d 0 control count. The mean percentage of macrocytic cells was increased at 45 mg/kg on d 7 and 10, but this increase was significant only at d 7. MCV fell slightly, from 51.4 fL on d 0, to 50.5 fL (d 2 and 4) or 50.6 fL (d 3), but was increased on d 7 (55.1 fL) and d 10 (54.2 fL), reflecting the altered reticulocyte percentage (Fig. 3.10).

The depression of the reticulocyte count, and its return to the initial level, followed after the change in femoral erythroid cell count by approximately one day (Fig. 3.11); this can be clearly seen by comparing the reticulocyte counts on d 2, 3, and 4 (Fig. 3.10) with the femoral marrow erythroid cells on d 2, 3 and 4 (Fig. 3.11). The reduction in the erythroid population, which was maximally depressed on d 3, at less than 2 % of the d 0 control value, showed the most marked effect on marrow cellularity (Fig. 3.12). This compared with reductions in the cells of the myeloid series on d 3 to 32 % of the d 0 control value

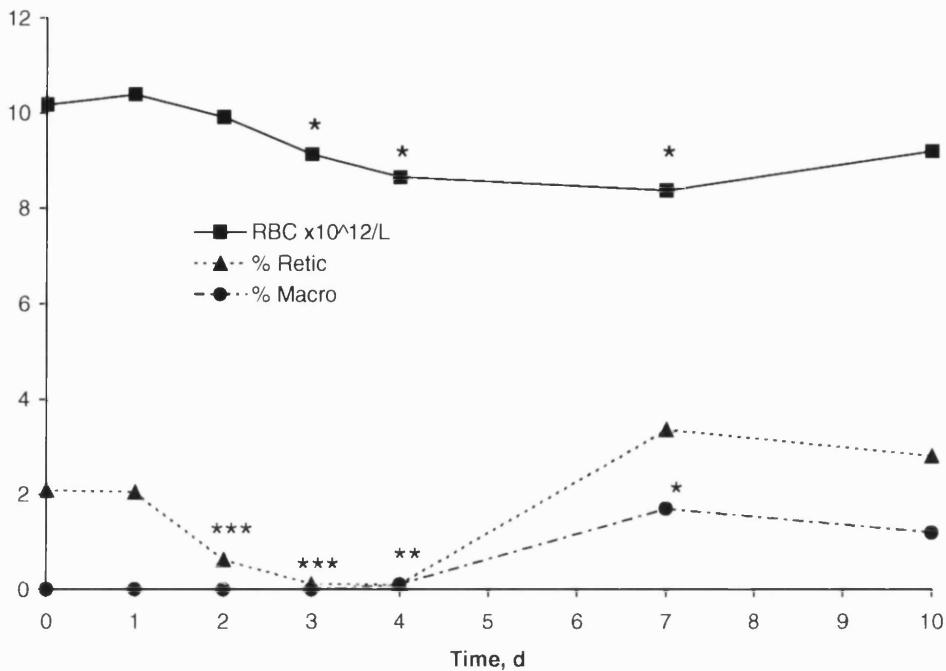


Figure 3.10: Mean erythrocyte count, and percentage reticulocytes and macrocytic cells following a single BU dose of 45 mg/kg. n=5; * significantly different to control value, p<0.05; **p<0.01; *p<0.001.**

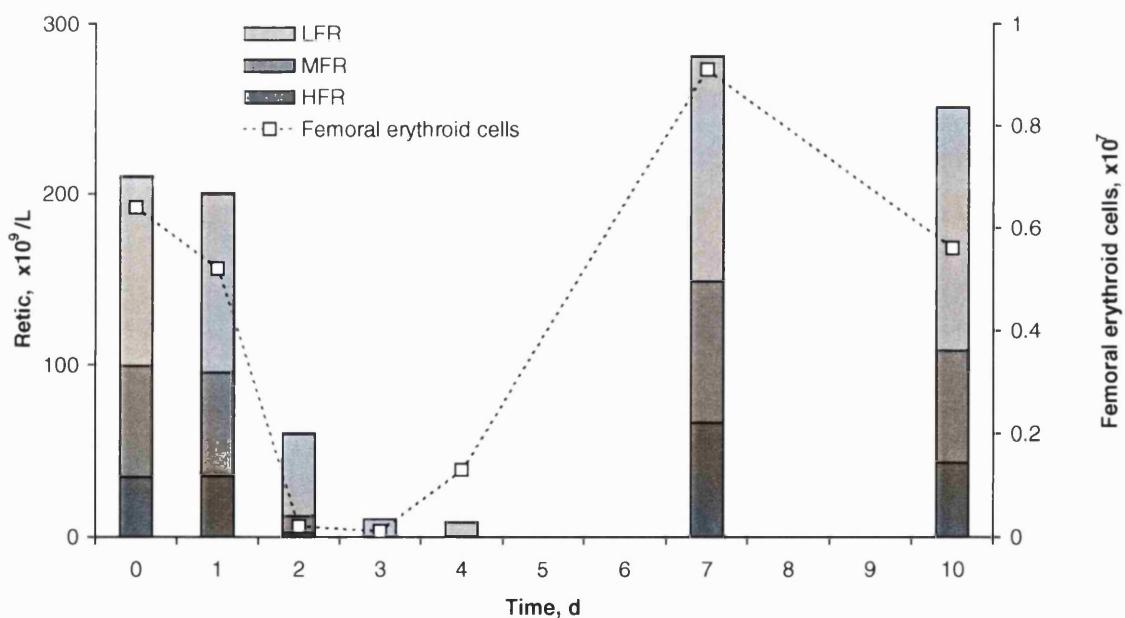


Figure 3.11: Mean femoral marrow erythroid precursor cells and absolute peripheral reticulocyte count following a single BU dose of 45 mg/kg. Reticulocytes are subdivided into low, mid- and high fluorescence fractions (LFR, MFR, HFR). n=5.

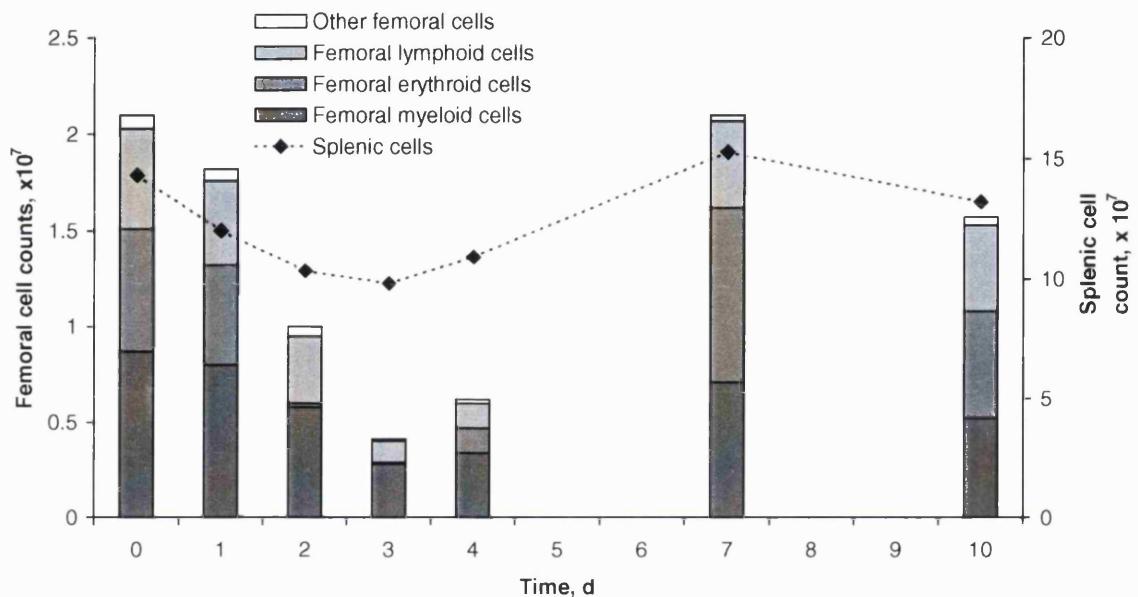


Figure 3.12: Mean femoral marrow and splenic nucleated cell counts following a single BU dose of 45 mg/kg. The proportions of femoral myeloid, erythroid, lymphoid and other cells are shown as shaded subdivisions of the total cell counts. Note that erythroid precursors are virtually absent by d 3. n=5.

and cells of the lymphoid series to 21 % (Fig. 3.12). Reduction in total femoral count at 45 mg BU/kg on d 3 was to 23 % of the d 0 control value (Fig. 3.12). Reduction in spleen cellularity (Fig. 3.12), and subsequent recovery, followed the same time course as the femoral cell count (Fig. 3.12), but the response of the spleen was proportionally less marked than the effect on the marrow, the maximum reduction in the spleen cell counts at d 3 being to 69 % of the d 0 value.

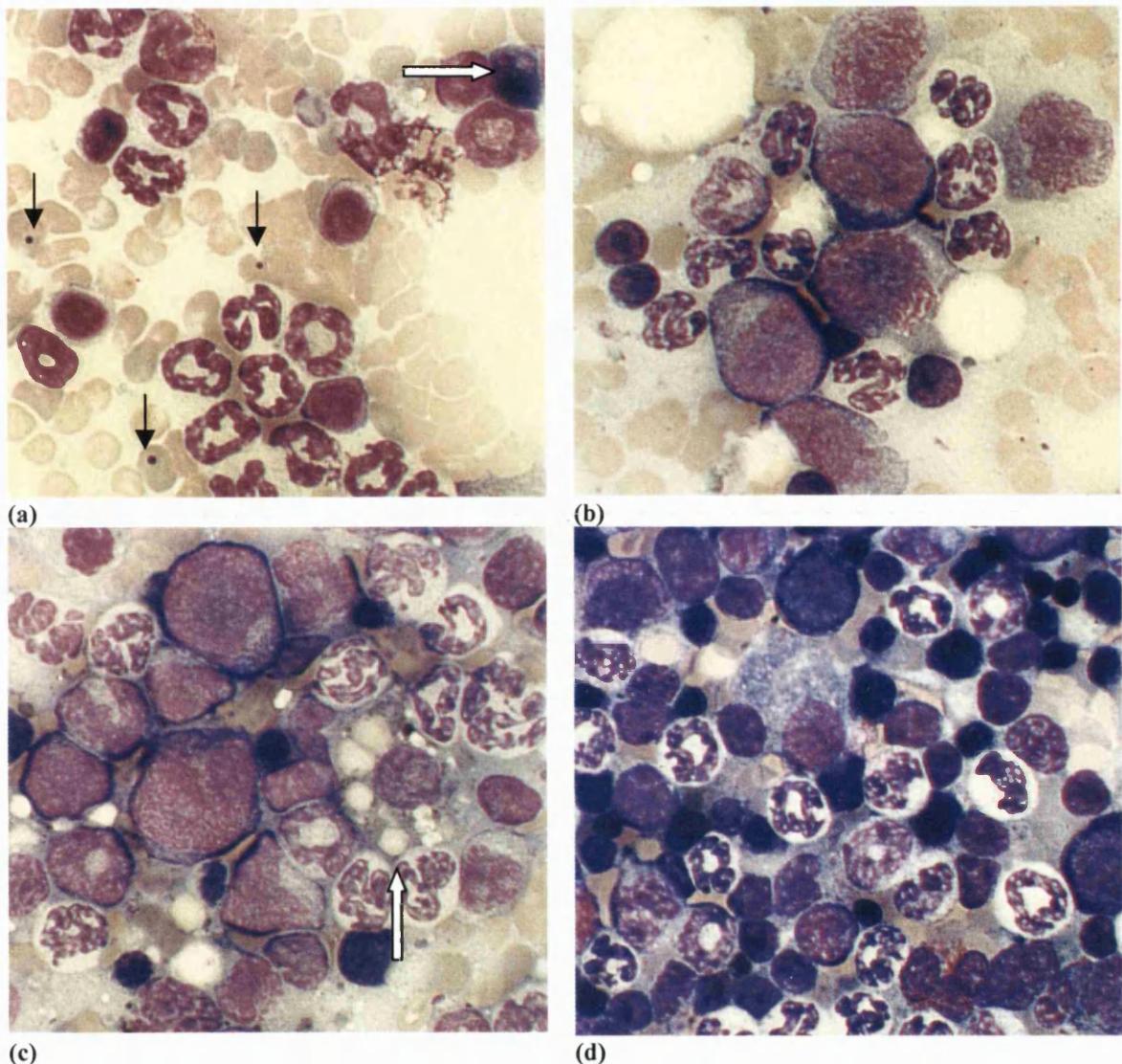


Figure 3.13: May-Grünwald-Giemsa stained femoral bone marrow smears of individual mice at different timepoints following a single dose of 45 mg BU/kg. Final magnifications $\times 1200$.

- (a) 2 d post dose. There is an absence of virtually all precursors of the erythroid series and of myeloid cells earlier than metamyelocytes. A multinucleated late normoblast (large arrow) and Howell-Jolly bodies (arrows) are present. TNC = 0.92×10^7 ; M:E = 62:1.
- (b) 3 d post dose. The marrow is markedly hypoplastic, but evidence of regeneration is seen. A group of promyelocytes and metamyelocytes are seen TNC = 0.58×10^7 ; M:E = 1.17:1.
- (c) 4 d post dose. Regeneration of myeloid and erythroid elements is clearly evident. Dysplastic changes, including nuclear hypersegmentation, are evident in cells of the myeloid series. Erytrophagocytosis is present (arrow). TNC = 0.45×10^7 ; M:E = 6.24:1.
- (d) 7 d post dose. The marrow is fully cellular and the morphology of the myeloid series appears normal, however, a predominance of erythroid elements persists. TNC = 2.45×10^7 ; M:E=0.53:1.

The relationship of femoral erythroid cell numbers and the maturity of peripheral reticulocytes is shown in Fig. 3.11. Femoral erythroid cells were reduced to 3.1 % of the d 0 control value on d 2, and whilst the total reticulocyte count was 28.6 % of the d 0 value at this time. HFR reticulocytes on d 2 were reduced to 5.8 % of the d 0 control. On d 3, these percentages had fallen further, to 1.6 % (femoral erythroid cells), 4.8 % (total reticulocytes) and 0.3 % (HFR reticulocytes), of the d 0 values. Recovery of erythropoiesis with overshoot is clearly evident at d 7 (Fig. 3.11), with femoral erythroid cells and total reticulocytes being 142 % and 133 % of d 0 values, respectively. However, in this recovery phase, the absolute HFR reticulocyte count showed the greatest increase, to 190 % of the d 0 value (Fig. 3.11).

The appearance of femoral marrow smears at various times after treatment with BU at 45 mg/kg is illustrated in Fig. 3.13. At d 2 there was marked hypocellularity with an absence of, or very marked reduction in, cells of the erythroid series (Fig. 3.13 (a)). The occasional late normoblast present showed dyserythropoietic changes. Myeloid cell numbers were reduced, and myeloblasts and promyelocytes were also absent at this time. Lymphoid cells were present in moderate numbers. Although the marrow smears were still markedly hypoplastic at d 3 and 4 (Fig. 3.13 (b) and (c)), in contrast to d 2, the majority of myeloid and erythroid cells present were primitive, that is, myeloblasts, promyelocytes, pronormoblasts and early normoblasts predominated. The nuclei of promyelocytes showed considerable pleiomorphism, and the presence of haemosiderin and erythrophagocytosis provided evidence of erythrocyte destruction. At d 7 (Fig. 3.13 (d)), normal cellularity of both myeloid and erythroid lineages had been restored, but both lineages were left-shifted, that is, immature cells were more numerous than in control smears. At d 10, smears appeared fully cellular and morphology and cellular distribution

of all lineages appeared normal. Megakaryocyte numbers appeared adequate at all timepoints, but at d 3 and later timepoints, megakaryocyte development also showed a left shift, that is, nuclear ploidy was reduced and the cytoplasm was more basophilic and less extensive, and granularity was less pronounced.

The effects on the erythrocyte series and the temporal relationship of its maturational components after 45 mg BU/kg are summarised in Fig. 3.14. The relative changes in femoral erythroid cells, and HFR and LFR reticulocytes closely parallel each other, but a clear lag can be seen between the individual responses of each of these three counts which is in keeping with their relative maturity. The maximal depression of erythrocyte count is seen at d 7, at which time the HFR count is most elevated. Due to the absence of samples on d 5 and 6 it is not known whether elevations of the femoral erythroid cell count to higher levels occurred on these days, but increases of a similar magnitude to that of HFR might have been expected.

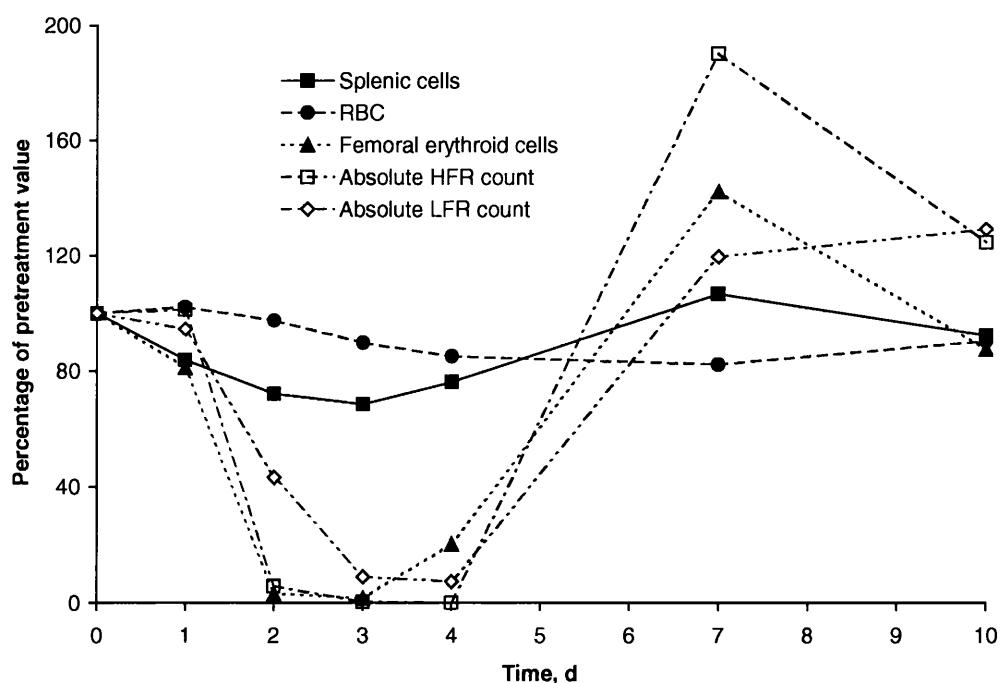


Figure 3.14: Summary of erythroid series changes following a single BU dose of 45 mg/kg. Data (mean values) are expressed as percentages of the d 0 control counts.

Neutrophils, monocytes and eosinophils were elevated on d 1 to 150 %, 176.9 % and 180 % respectively, of the d 0 value following a single BU dose of 45 mg/kg (Fig. 3.15). The neutrophil and monocyte counts fell on d 2, and at d 4 were maximally depressed, to 31.7 % and 30.8 % of the d 0 values, respectively. However, eosinophils rose to 200 % of the d 0 value on d 2, but fell to 30 % on d 3. Thereafter, neutrophils, monocytes and eosinophils remained depressed until 10 d post dosing. LUC counts, on the other hand, fell immediately after dosing, to 82.6 % of the d 0 value. LUC then showed a marked rise starting on d 4, reaching a maximum of 156 % of the d 0 value at d 7 post dosing. Lymphocyte counts were slightly increased on d 1, to 112.3 % of the d 0 value, thereafter falling to a nadir of 58.8 % at d 4 and rising to 94.4 % of the d 0 value at d 10. Neutrophil counts and peroxidase content (MPXI) are displayed together in Fig. 3.16. MPXI showed an overall increase to d 7, whilst over the same period of time, there was a trend for reduction in the neutrophil count. At d 10, MPXI returned to the pretreatment value, but neutrophil count remained unchanged from the d 7 value.

No clear pattern in platelet counts emerged. Counts in animals receiving 35 and 45 mg BU/kg were reduced to (respectively) 67 % and 45 % of the d 0 value on d 3, and to 50 % and 48 % of the d 0 value at d 10 after dosing. At all other times, counts for BU-treated animals were similar to the control value.

Data from the mice treated with BU at 35 mg/kg is not shown. At 35 mg BU/kg, the patterns of change in cells of all lineages in the marrow, spleen and peripheral blood were similar to, but of a smaller magnitude than, the changes seen in these cell types at 45 mg/kg. However, both the absolute HFR and LUC counts showed a rebound on d 4 to 173.8 % and 130.4 % of their respective pretreatment values.

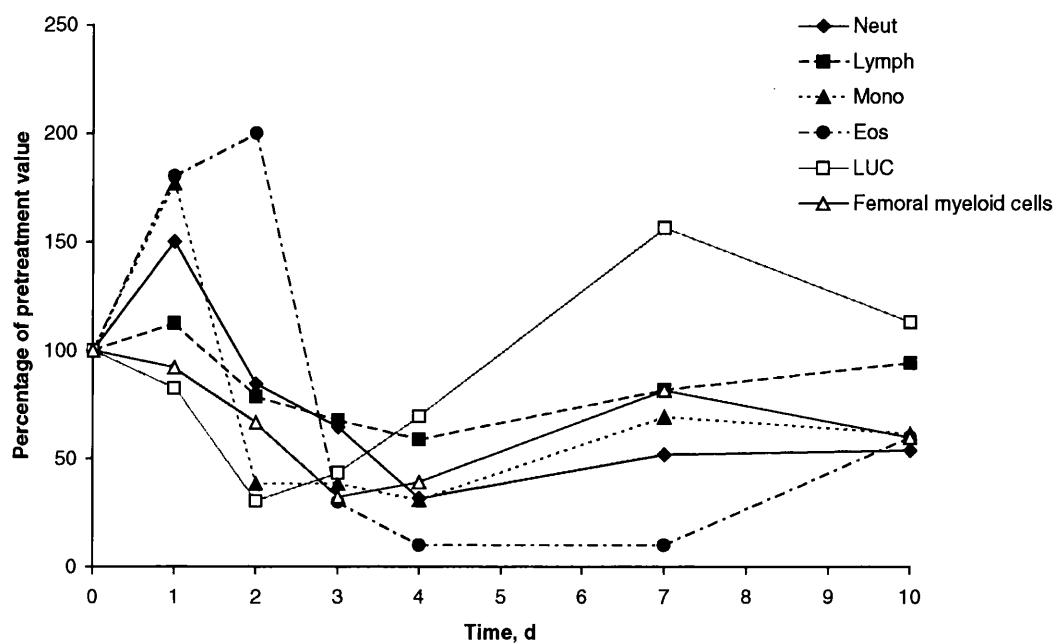


Figure 3.15: Mean leucocyte and bone marrow myeloid cell count following a single BU dose of 45 mg/kg. Data are expressed as percentages of the d 0 control counts.

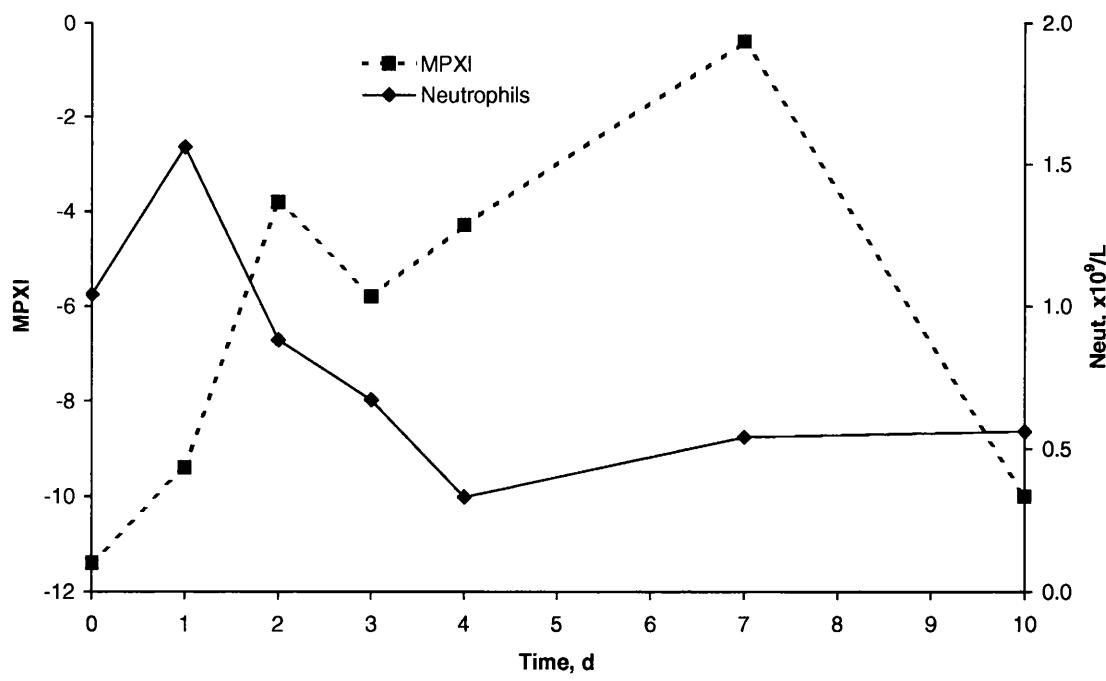


Figure 3.16: Mean neutrophil count and MPXI following a single BU dose of 45 mg/kg.

3.4 Discussion

In Experiment 1, single doses of BU were well tolerated up to a maximum of 45 mg/kg (Andrews *et al.*, 1992; 1993a). At 14 d after dosing, when it was considered that a repeat dose of BU would be given, slight treatment-related macrocytosis was present although there was no significant alteration in reticulocyte counts. Alterations to the proportion of macrocytes counted by the H*1 analyser usually indicate changes in the proportion of reticulocytes, which are slightly larger than mature RBC. This is particularly so in inbred rodents, in which the MCV shows little inter-individual variation, other than that due to the animal's age (Andrews, personal observation). However a correlation between macrocytosis and reticulocytosis is not necessarily inevitable (Bain and Cavill, 1993). Macrocytosis without an increase in reticulocytes may be due to megaloblastic RBC development as a result of disturbed DNA synthesis, and this response has been observed in mice given 1,3-butadiene (Irons *et al.*, 1986), AZT (Scheding *et al.*, 1994) and benzene (Farris *et al.*, 1997).

Neutrophil, monocyte and eosinophil counts were reduced at higher BU dose levels in Experiment 1 and this change is consistent with earlier reports (Elson, *et al.*, 1958; Dunn and Elson, 1970; Udupa *et al.*, 1972; Dunn, 1974; Delmonte, 1978; Kato *et al.*, 1988). However, at lower dose levels, elevations of neutrophil and lymphocyte counts were seen. Early stimulation of neutrophils was also seen in the mouse (Kato *et al.*, 1988) and rat (Delmonte, 1978) with low dose BU treatment, and it has been proposed that low dose BU may enhance granulopoiesis by synergy with GM-CSF (Irons and Stillman, 1993). Platelet counts in Experiment 1 showed a marked dose-related reduction. As all the above effects were quite marked at 45 mg/kg, especially the reduction in platelet counts, and bearing in mind the finding of moderate anaemia in one animal in the group of four, the

maximum dosage of BU selected for repeat administration (Experiment 2) was 40 mg/kg. However, for the characterisation of the short-term response to a single dose (Experiment 3), doses of 35 and 45 mg/kg were selected.

After four fortnightly BU doses (Experiment 2) the pattern of changes in cell counts was similar to those seen in Experiment 1 after a single dose, but the changes were of slightly greater magnitude (Andrews *et al.*, 1992; 1993a). The alterations in cell counts followed similar patterns at both 14 and 42 d post dosing, but at 42 d, with the exception of lymphocyte counts, cell counts appeared to be returning to normal. An apparent stimulation of lymphocyte counts was present at 14 d (Fig. 3.8), as was seen after a single BU dose in Experiment 1 (Fig. 3.3). As in Experiment 1, platelet counts were significantly depressed in Experiment 2 at all dose levels, and a dose-related decreasing trend was also evident. The magnitude of these cytopenias, and the absence of adverse clinical findings, suggested that a range of BU doses up to 40 mg/kg would be suitable for use in combination with CAPS dosing in later investigations (see Chapter 5).

The effect of a single ip dose of BU (Experiment 3) was to rapidly deplete morphologically recognisable femoral erythroid precursors and peripheral reticulocytes (Andrews *et al.*, 1993a, b). Total femoral and splenic cell counts were similarly reduced but recovered in the same time period. It has recently been reported that reduction in splenic cellularity in mice following BU administration is almost wholly due to loss of non-T lymphocytes (Szebeni *et al.*, 1997). Recovery of erythropoiesis, with an overshoot of immature (HFR) reticulocytes, was evident one week after dosing. However, as has been demonstrated with splenectomised mice (Blackett and Millard, 1976), the spleen contributes little to the recovery of erythropoiesis immediately after BU treatment. The

rapidity of this erythroid recovery response is in keeping with a direct toxicity of BU to the committed erythroid precursors BFU-E and CFU-E (Delmonte, 1978; Boggs and Boggs, 1980). It would also indicate that although BU may have damaged earlier pluripotent stem cells (Dunn and Elson, 1970; Morley *et al.*, 1975), a sufficient reserve remained for lineage commitment and differentiation to make up the deficit of mature cells.

The usefulness of studying reticulocyte maturation has been advocated in human patients as an indicator of marrow recovery after transplantation (Davis *et al.*, 1989; Davies *et al.*, 1992) and following chemotherapy (Jouault *et al.*, 1992; Kuse *et al.*, 1996). The present findings show that HFR, which constitute a much larger fraction of reticulocytes in the mouse than in man (Fuchs and Eder, 1991; Hickson, 1992), may be used as a sensitive indicator of marrow recovery in experimental models.

The morphologically recognisable femoral myeloid component was initially reduced by BU treatment in Experiment 3 (Fig. 3.12, 3.13), consistent with toxicity to CFU-GM. There was then a gradually recovery in myeloid cells but to less than 60 % of the d 0 control value. However, additionally, there were increases of peripheral granulocytes (neutrophils and eosinophils) and monocytes in the first 1-2 d post dosing (Fig. 3.15). Eosinophilia is seen in mice after treatment with another cytotoxic agent, CY, although the peak in eosinophil count occurs at 14 d after CY administration (Sugane and Oshima, 1985). The increase in neutrophil MPXI was a consistent finding in all three experiments. Increases in MPXI have been reported in megaloblastic anaemia (Gulley *et al.*, 1990) and following hydroxurea therapy (Froom *et al.*, 1989). Both in megaloblastic anaemia, and following hydroxyurea therapy, DNA synthesis is perturbed, as is seen following BU

administration (Dunn, 1974; Hall and Tilby, 1992). Similarly, in both conditions erythrocyte macrocytosis is present (Price *et al.*, 1985), as was seen in the present series of experiments. In addition, megaloblastic change was evident in myeloid precursors in Experiment 3 (Fig. 3.13 (c)). Nevertheless, Froom *et al.* (1989) reported no change in MPXI in patients treated with other antineoplastic agents which inhibit DNA synthesis, such as methotrexate, and there was a marked decrease in MPXI in one of four patients receiving BU. Increases in MPXI have been associated with toxic granulation of neutrophils (Peacock *et al.*, 1982) and precede rising circulating concentrations of myeloperoxidase (Zipfel *et al.*, 1997), but do not correlate with morphological signs of neutrophil immaturity (Peacock *et al.*, 1982; Tsakonas *et al.*, 1994). In addition, it should be noted that haemoglobin itself possesses pseudoperoxidase activity, and the decreased haemoglobin concentration of a blood sample may result in elevated MPXI due to decreased consumption of the enzyme substrate (Hewitt and Reardon, 1991).

The recovery of peripheral leucocytes in Experiment 3 was preceded by a marked increase in the LUC count. Leucocytes larger than lymphocytes, but having lower peroxidase activity than monocytes are designated large unstained cells (LUC) by the H*1 haematology analyser. In health, LUC are lymphocytes and monocytes in approximately equal proportions (Kinsey and Watts 1988). However, LUC may correspond also to leukaemic blasts or lymphoma cells (d'Onofrio *et al.*, 1987; Kline *et al.*, 1989) or atypical mononuclear cells preceding recovery from marrow aplasia (Kinsey *et al.*, 1989). Mice treated with FL, which has been demonstrated to mobilise haemopoietic progenitor cells (Ashihara *et al.*, 1998) show marked increases in LUC counts, and these precede increases in neutrophil, monocyte and lymphocyte counts (Juan *et al.*, 1997).

In Experiment 3 the effect on platelet counts after a single dose of BU was generally inconsistent at both 35 and 45 mg/kg, although clear reductions in counts were noted at some timepoints.

In summary, a single BU dose at 45 mg/kg caused an initial depletion of erythrocyte precursors which then showed an almost complete recovery after 14 d (Andrews et al., 1992; 1993a; 1993b). The HFR count was a sensitive indicator of erythroid recovery. However, alteration of erythroid maturation was indicated by slight but significant increases in macrocytic cells. Repeated doses of BU at 40 mg/kg caused a mild anaemia with slight macrocytosis which was not fully compensated. Reductions in femoral myeloid and lymphoid cells were seen after a single BU dose, but these reductions were to a lesser degree than that seen in erythroid cells. An initial increase in granulocytes and monocytes after BU dosing was followed by decreases of all leucocytes. The BU-induced reduction in neutrophil count was associated with an increase in MPXI, but thereafter a marked rise in LUC counts suggested a return towards normal levels of granulopoiesis, although myeloid precursors were still decreased after 10 d. Reductions of granulocytes and slight increases in neutrophil MPXI were still present at two weeks (d 14) and six weeks (d 42) after repeated BU doses. Lymphocyte counts had returned to normal at 10 and 14 d after a single BU dose at 35 to 45 mg/kg, but at lower dose levels the counts were increased at 14 d. Slight, but non-significant lymphocyte increases were also present at the lower BU dose levels at two weeks after repeat dosing, but at 30 to 40 mg BU/kg the lymphocyte count still showed significant depression after six weeks. Dose-dependent reductions in platelet counts were seen following a single BU dose. A dose-related trend in platelet reduction persisted at two and six weeks after repeat dosing, and reductions in

counts were seen at all dose levels. In B6C3F₁ mice which received four fortnightly doses of BU at 35 to 45 mg/kg, slight but persistent cytopenias of all lineages were present six weeks post dosing, although there was evidence that counts were improving. All animals treated with four fortnightly doses of BU at up to 45 mg/kg were clinically well at six weeks post dosing, and it was therefore considered that for subsequent CAPS treatment, animals should be pretreated on four occasions with BU at 40 mg/kg.

CHAPTER 4: CHLORAMPHENICOL SUCCINATE ADMINISTRATION IN THE DRINKING WATER: DOSE RANGING STUDIES AND HAEMATOLOGICAL EFFECTS

4.1 Introduction

A dose level of 5 mg CAPS/mL was used in the drinking water of mice previously treated with BU by Morley *et al.* (1976b). This level was subsequently used by Pazdernik and Corbett (1980). Robin *et al.* (1981) administered approximately 100mg CAP/kg, 5 d per week, for 5 weeks, by sc injection. However, calculations show that the level of CAPS in the drinking water employed by Morley *et al.* (1976b) would potentially deliver more than 400 mg CAPS/kg/d. Although CAP palmitate (as a suspension) is normally used in man for oral administration (Parfitt, 1999), CAP palmitate is relatively insoluble, and therefore the succinate form of CAP, which is more soluble and commonly used therapeutically for injections, was used for administration in drinking water in the present studies, as originally proposed by Morley *et al.* (1976b). Orally-administered CAPS is rapidly hydrolysed *in vivo* in the gastric mucosa to CAP (Nahata and Powell, 1983), however, 30 % of an iv or intramuscularly administered CAPS dose is excreted renally in man (Ambrose, 1984). When CAPS was given in the drinking water to mice at 5 mg/mL, serum concentrations of CAP of between 2.5 and 4.0 µg/mL were obtained (Morley *et al.*, 1976b). This level was judged by these workers to be sufficient to cause haemotoxicity, as a 50 % inhibition of murine CFU-C had been reported at levels of 1 to 10 µg/mL (Morley *et al.*, 1974a). Nevertheless, marked variations in the *in vitro* sensitivity to CAP between differing strains of mouse have also been reported (Miller *et al.*, 1978). Therefore, the predictions of palatability and toxicity of CAPS when administered in the drinking water, from the BALB/c mouse (Morley *et al.*, 1976b) to the B6C3F₁ mouse, to be used in Experiments 4 and 5, might not hold. It was therefore necessary to carry out

comprehensive dose ranging studies on the palatability and toxicity of CAPS administered in the drinking water to B6C3F₁ mice.

Two studies on the effects of CAPS administered in the drinking water were carried out.

First, CAPS was to be administered at six concentrations in the drinking water, and palatability and other effects assessed in a study lasting seven days (Experiment 4). In the second study (Experiment 5), two CAPS levels would be administered in an investigation lasting 30 days. The selected dose range (1.0 - 6.0 mg CAPS/mL of drinking water) for the seven-day experiment (Experiment 4) was based on the original level of 5 mg/mL used by Morley *et al.* (1976b) in BALB/c mice. The aim of this investigation was therefore to establish the maximum tolerated dose (MTD) of CAPS in the B6C3F₁ mouse when administered in the drinking water. The assessment of dose toleration would be based on the palatability of the CAPS solution in drinking water as indicated by drinking water consumption and clinical signs. In the 30 d study (Experiment 5) the MTD and a lower level would be used to assess myelotoxicity over a longer period. In both experiments, detailed haematological analysis would be performed to assess effects on haemopoiesis.

4.2 Materials and Methods

Mice were obtained from the Small Animal Breeding Unit, Glaxo Wellcome Research and Development, Ware, Herts. CAPS administration in the drinking water and blood sampling and analysis were as described in Chapter 2.

4.2.1 Experiment 4: Determination of the Maximum Tolerated Dose of Chloramphenicol Succinate in the Drinking Water: A Seven-day Study

Seven groups of 10 mice were given drinking water only or drinking water containing CAPS at a final concentration of 0, 1, 2, 3, 4, 5 and 6 mg/mL *ad libitum* for 7 d. Animals were observed daily, killed on d 7 and full blood count, differential leucocyte count and reticulocyte count performed. Water consumptions and body weights were measured daily.

4.2.2 Experiment 5: Haematological Effects of Chloramphenicol Succinate in the Drinking Water: a 30-day Study

Three groups of 30 mice were given drinking water only or drinking water containing CAPS at a final concentration of 0, 2.5 or 4.0 mg/mL *ad libitum* for 30 d. Five animals per group were killed after 5, 10, 15, 20, 25 and 30 d dosing and full blood count, differential leucocyte count and reticulocyte count performed. Water consumptions were measured daily and body weights twice weekly.

4.3

Results

4.3.1 Experiment 4: Determination of the Maximum Tolerated Dose of Chloramphenicol Succinate in the Drinking Water: A Seven-day Study

4.3.1.1 Clinical Observations, Water Consumption and Body Weights

Animals appeared well throughout the study and there were no clinical signs of toxicity.

However, marked reductions in water consumption were evident each day after d 1

($p<0.001$) in animals receiving 3.0 mg CAPS/mL and above (Fig. 4.1). Body weights

showed significant reductions at 4.0 mg CAPS/mL and above (Fig. 4.2); these reductions

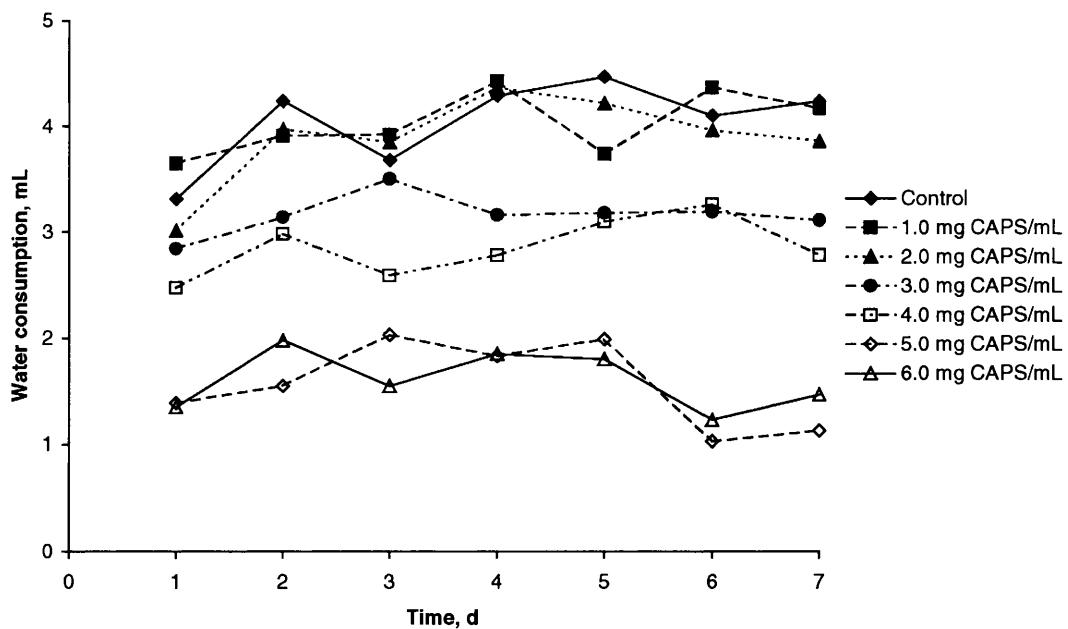


Figure 4.1: Mean water consumption per mouse per day in mice receiving CAPS at the levels indicated in the drinking water for 7 d. $n=10$; the mean daily consumption over the 7 d period in groups receiving 3.0, 4.0, 5.0 and 6.0 mg CAPS/mL was significantly different from the control value ($p<0.001$); groups receiving 1.0 and 2.0 mg/mL, ns.

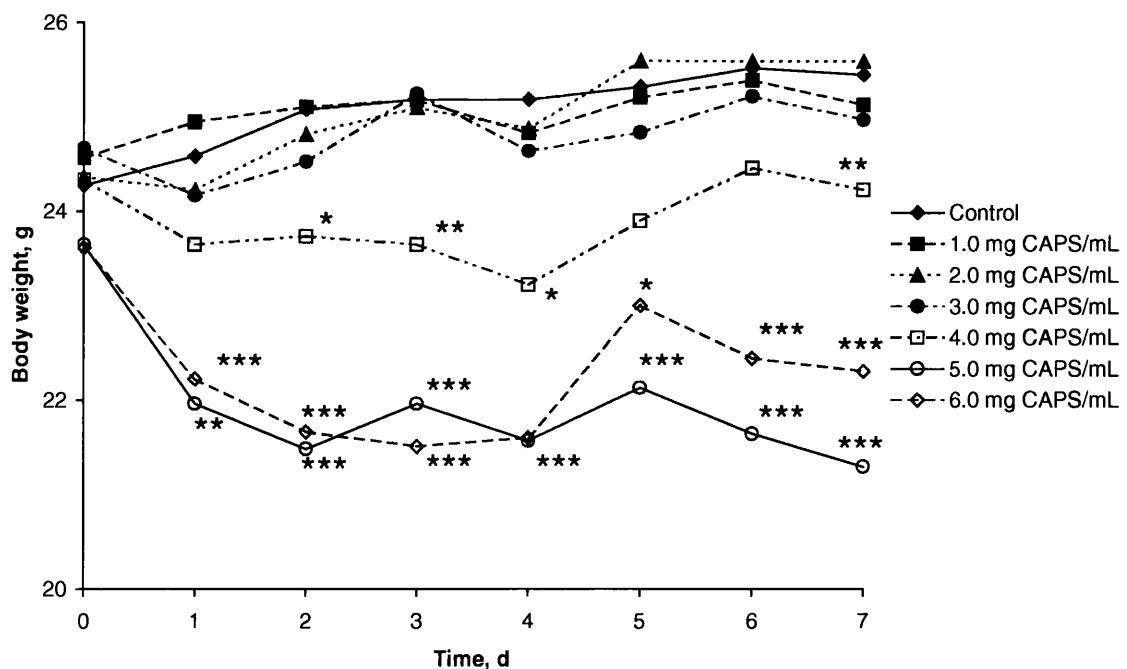


Figure 4.2: Mean body weights in mice receiving CAPS in drinking water at the concentrations indicated for 7 d. n=10; *significantly different to control value, p<0.05; **p<0.01; *p<0.001.**

were most marked in animals receiving 5.0 and 6.0 mg CAPS/mL, where they were apparent from the first day of dosing onwards. The calculated mean daily consumption of CAPS per mouse is shown in Table 4.1.

Table 4.1: Mean daily consumption of CAPS in mice receiving CAPS in the drinking water for seven d at the levels indicated.

Concentration of CAPS in drinking water, mg/mL	Mean daily CAPS consumption per mouse, mg/kg
0	0
1.0	160.9
2.0	312.0
3.0	383.2
4.0	478.8
5.0	355.9
6.0	433.9

4.3.1.2 Haematology

Erythrocyte count, haemoglobin concentration and haematocrit showed dose-related increases after the 7 d CAPS dosing; these changes were highly significant at the 5.0 and 6.0 mg CAPS/mL dose levels (Fig. 4.3). The mean increases in erythrocyte count were of a similar magnitude at both dose levels: 6.3 % at 5.0 mg/mL and 7.1 % at 6.0 mg/mL. Reticulocyte counts were depressed significantly at 3.0 mg CAPS/mL and above. There was no effect on MCV, RDW, HDW, percentage microcytes or macrocytes. No significant changes were observed in any other haematological parameter.

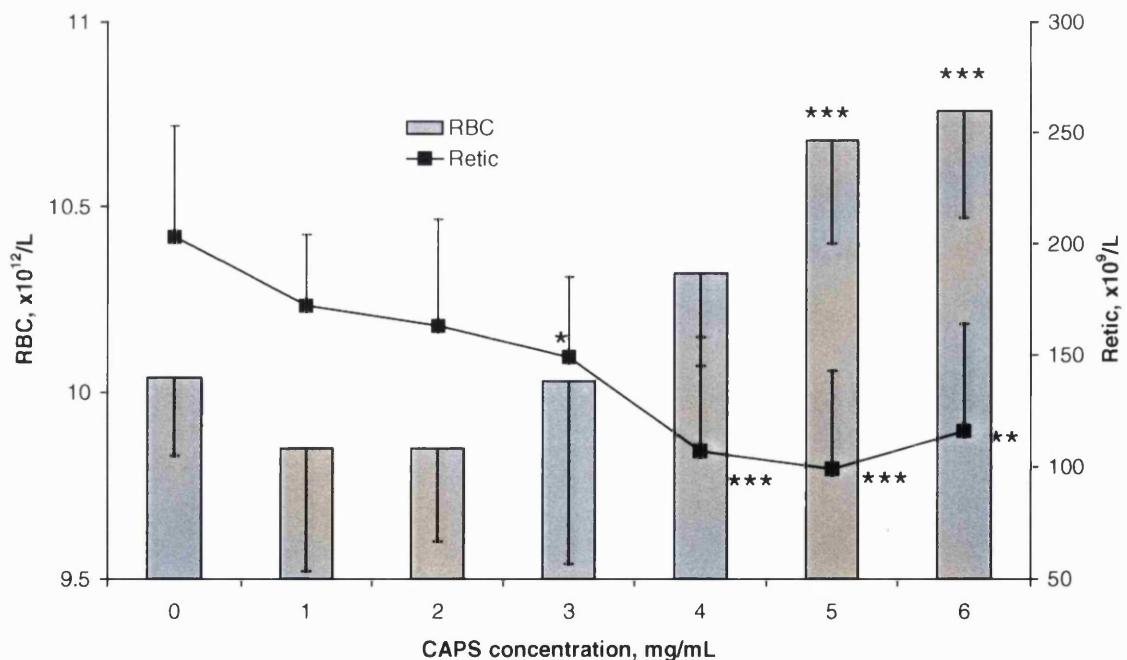


Figure 4.3: Mean and sd of erythrocyte and reticulocyte counts in mice receiving CAPS in drinking water for 7 d. n=10; * significantly different to control value, p<0.05; ** p<0.01; * p<0.001.**

4.3.2 **Experiment 5: Haematological Effects of Chloramphenicol Succinate in the Drinking Water: a 30-day Study**

4.3.2.1 Clinical Observations, Water Consumption and Body Weights

Animals appeared well at all times, and there were no signs of toxicity at either the 2.5 or 4.0 mg CAPS/mL dose levels. However, the mean daily water consumption per mouse was reduced at both dose levels over the 30 d period (Fig. 4.4), being 4.19 (sd 0.57) mL for controls, 3.78 (sd 0.50) mL for animals receiving drinking water containing 2.5 mg CAPS/mL ($p<0.05$), and 2.94 (sd 0.44) mL for animals receiving 4.0 mg CAPS/mL ($p<0.001$). This dose-related reduction in drinking water consumption remained approximately constant over the whole period of the experiment at both CAPS dose levels. The individual daily CAPS consumption per mouse, based on the water consumption data, was 360.1 mg CAPS/kg/d for the 2.5 mg/mL group and 439.8 mg/kg/d for the 4.0 mg/mL group. Body weights were not affected in the 2.5 mg CAPS/mL group, but were significantly reduced on d 5 and 8 in animals receiving 4.0 mg CAPS/mL ($p<0.05$), but thereafter were not different to the control group (Fig. 4.5).

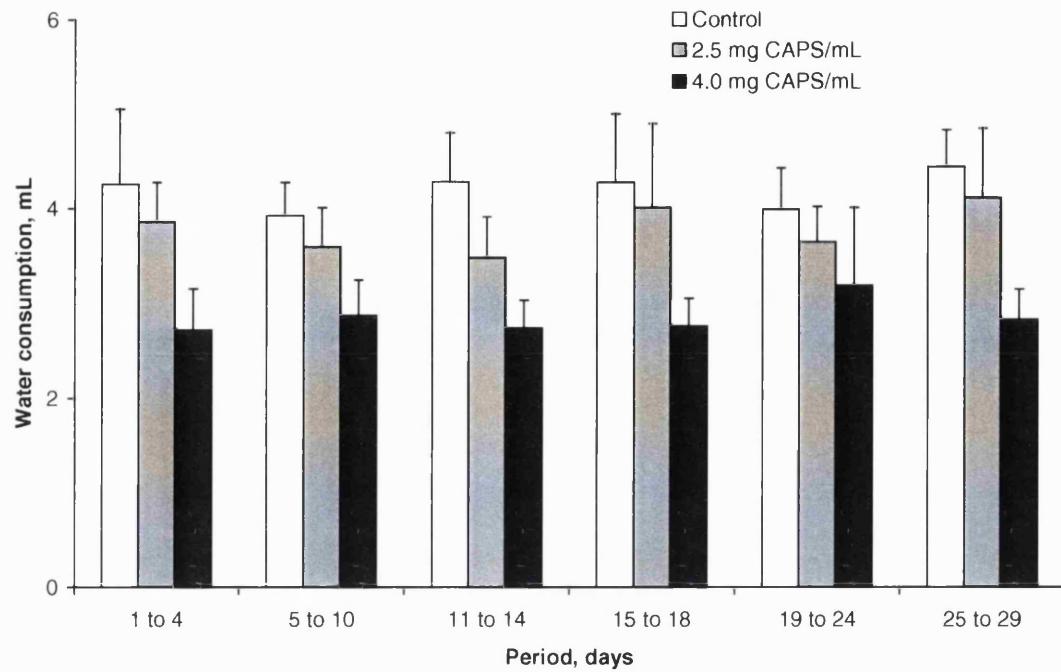


Figure 4.4: Mean and sd of daily water consumption per mouse receiving water alone (control) or CAPS in the drinking water at 2.5 and 4.0 mg/mL. Animals receiving 4.0 mg CAPS/mL consumed significantly less than controls ($p<0.001$) in all periods except d 19 - 24; 2.5 mg CAPS/mL, ns at all time periods. $n=6$ cages per group for d 1 to 4, reducing by 1 cage for each subsequent period.

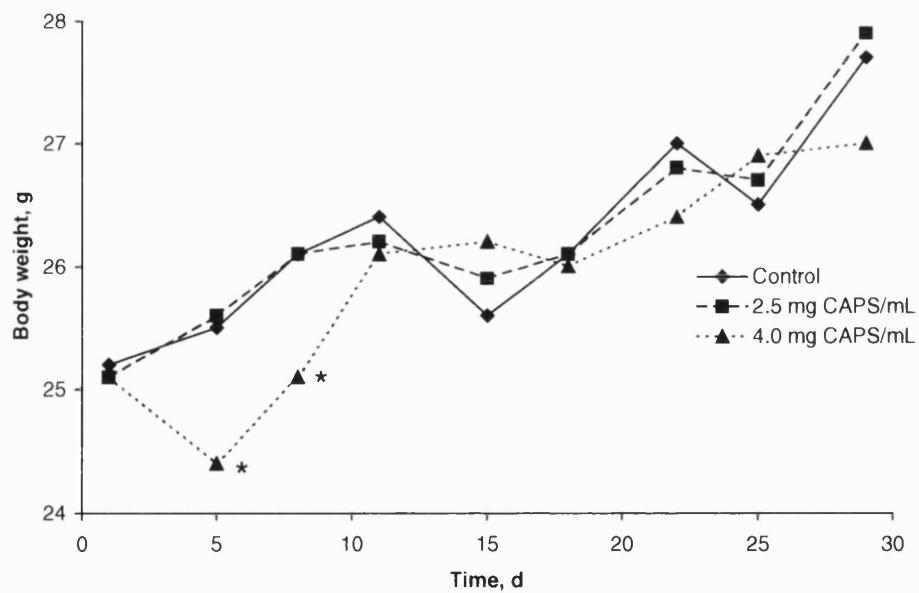


Figure 4.5: Mean body weights of mice receiving tap water (control) or CAPS in the drinking water at 2.5 and 4.0 mg/mL for up to 30 d. $n=30$ on d 5, reducing by 5 at d 10, 15, 20, 25 and 30; * significantly different to control value, $p<0.05$.

4.3.2.2 Haematology

The mean erythrocyte count for animals receiving 4.0 mg CAPS/mL was significantly elevated by 5.8 % ($p<0.05$) above the control value on d 5, but thereafter no significant changes were evident (Fig. 4.6). Reticulocyte counts in this group were depressed on d 5 and 10, rising to a value of 148 % of the control count on d 15, after which they were not significantly different from the control value (Fig. 4.7). In the 2.5 mg/mL group, erythrocyte counts were unaffected (Fig. 4.6). However, reticulocytes were depressed on d 5 at 2.5 mg CAPS/mL (Fig. 4.7), but to a lesser extent than in the 4.0 mg/mL group. Reticulocyte counts in the 2.5 mg/mL group on all other occasions were close to the control value. HFR counts showed a similar pattern to the total reticulocyte counts in both dose groups. The relationship of body weight, erythrocyte and reticulocyte counts in animals receiving 4.0 mg CAPS/mL is shown in Fig. 4.8. At d 5, the significant elevation of the erythrocyte count coincided with significant depressions of body weight and reticulocyte count. At d 8, body weight was returning to normal, and at d 10, the reticulocyte count, although still significantly depressed, was also closer to the control value. The erythrocyte count was normal at d 10 and body weight was normal at d 11, both remaining so until d 30, but the reticulocyte count overshot at d 15, before returning to normal at d 20.

Platelet counts in the 4.0 mg/mL group were significantly depressed on d 5, but were not significantly different from the control values at other times. In the 2.5 mg CAPS/mL group, platelet counts were unaffected. Other haematological measurements were unaffected by CAPS treatment.

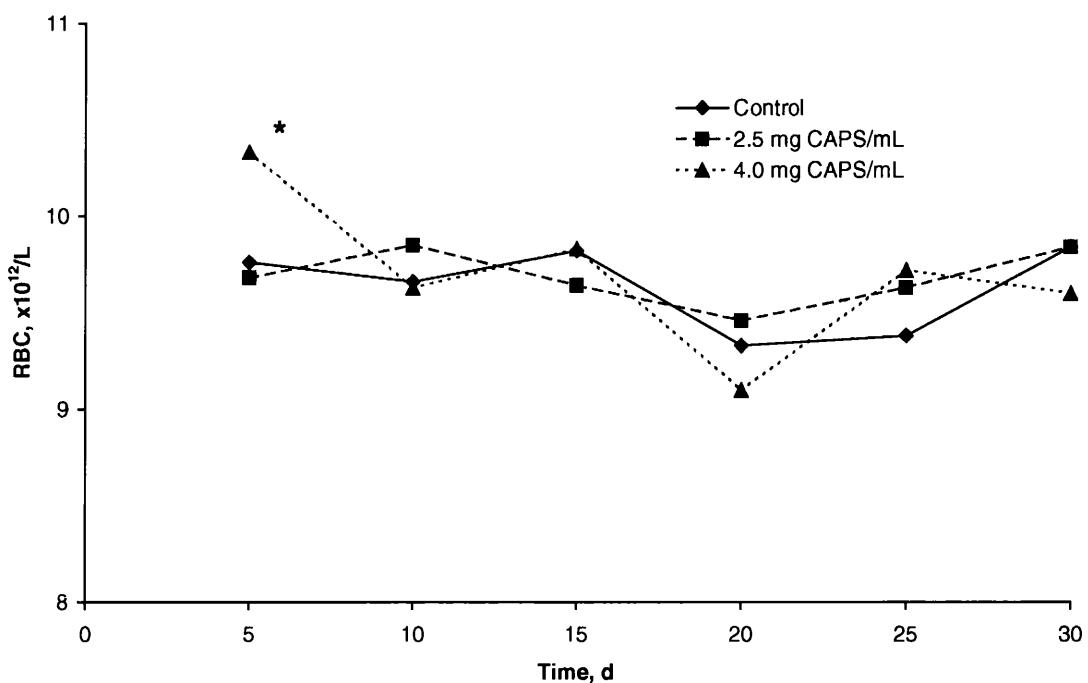


Figure 4.6: Mean erythrocyte counts in mice receiving tap water (control) or CAPS in the drinking water at 2.5 and 4.0 mg/mL for up to 30 d. n=5; *significantly different to control value, p<0.05.

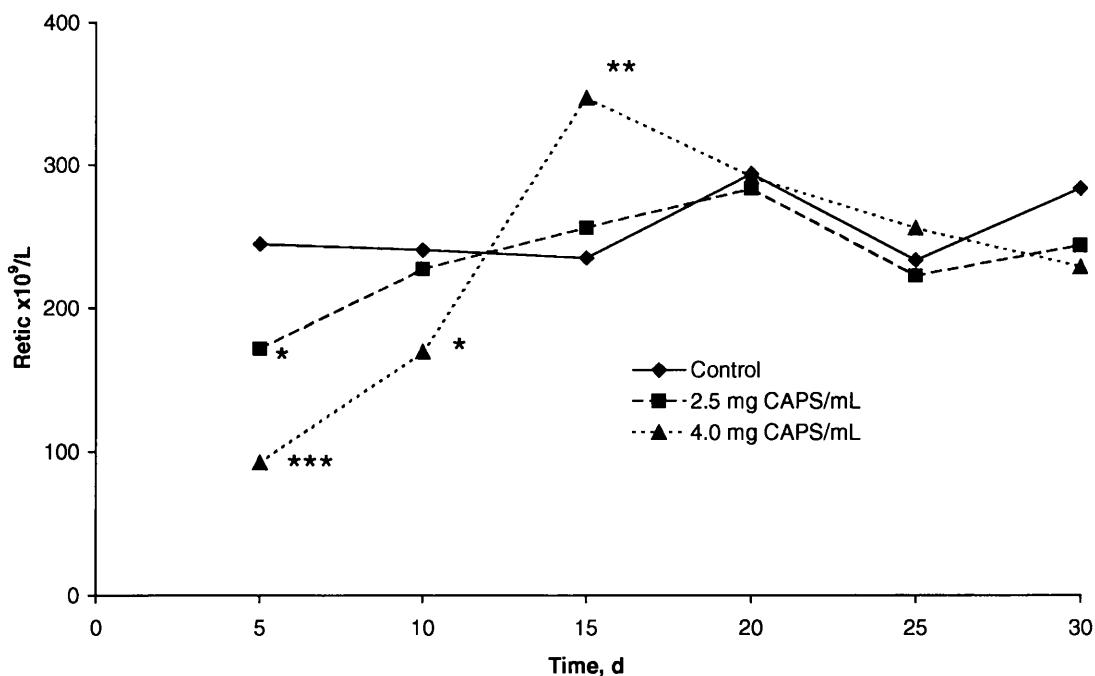


Figure 4.7: Mean reticulocyte counts in mice receiving tap water (control) or CAPS in the drinking water at 2.5 and 4.0 mg/mL for up to 30 d. n=5; *significantly different to control value, p<0.05; **p<0.01; ***p<0.001.

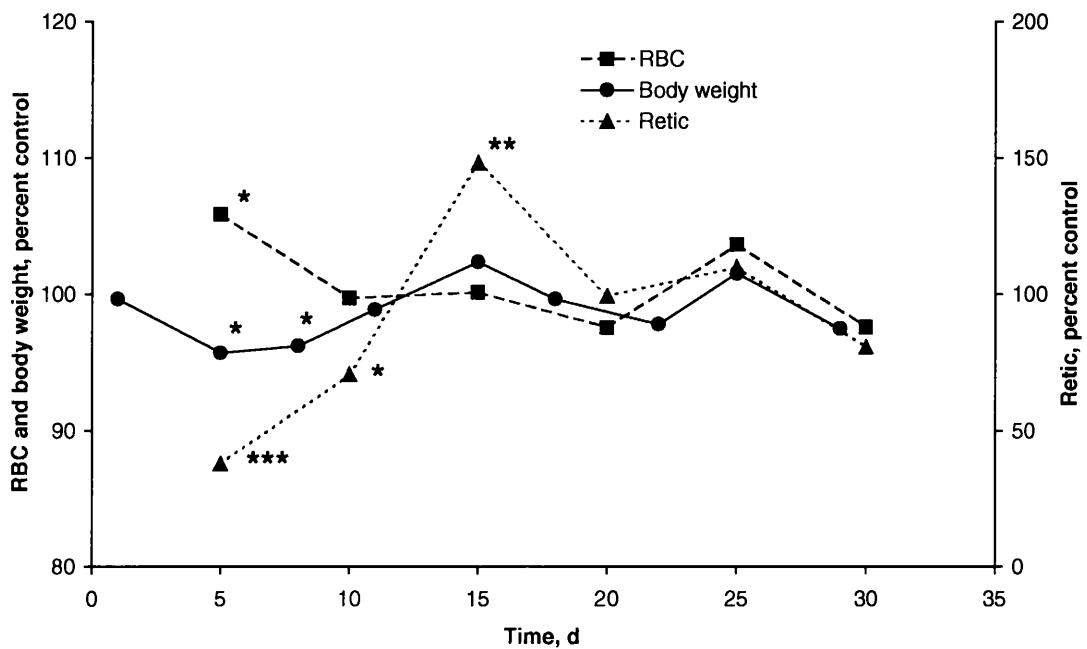


Figure 4.8: Mean erythrocyte count, reticulocyte count and body weights expressed as percentages of time-matched control values in mice receiving 4.0 mg CAPS/mL in the drinking water for up to 30 d. n=5; *significantly different to control value, p<0.05; ** p<0.01; * p<0.001**

4.4 Discussion

CAPS was not palatable to B6C3F₁ mice at levels above 4.0 mg/mL when administered in the drinking water for one week (Experiment 4). At levels of 3.0 mg CAPS/mL and above, there was reduced water consumption (Fig. 4.1). At 4.0 mg/mL and above, there was significant loss of body weight (Fig. 4.2) and depression of the reticulocyte count (Fig. 4.3), and at 5.0 and 6.0 mg CAPS/mL, the erythrocyte count was significantly elevated (Fig. 4.3).

When CAPS was given for 30 d at 4.0 mg/mL (Experiment 5) it did not appear to exert any myelotoxicity at the later stages of the the experiment. These findings are in keeping with other reports of CAP and CAPS treatment in mice: Morley *et al.* (1976b) gave 5.0

mg/mL in the drinking water and reported no effect on marrow cellularity, CFU or CFU-GM. Pazdernik and Corbett (1980) detected no change in femoral cellularity with CAPS administered by the same route and at the same dosage as Morley *et al.* (1976b), although femoral CFU-GM, splenic cellularity and splenic antigen-reactive cells were reduced after six weeks' CAPS administration. Robin *et al.* (1981) reported no effect on peripheral counts with CAP alone when given ip at 100 mg/kg, 5 d/week for 5 weeks. Nevertheless, the reports of Morley *et al.* (1976b), Pazdernik and Corbett (1980) and Robin *et al.* (1981) all indicate that the haemotoxic effects of BU treatment may be influenced by subsequent CAP or CAPS administration.

In view of the marked effect of CAPS on body weight in Experiment 4, and the moderate reduction in reticulocyte count seen at 5.0 and 6.0 mg CAPS/mL, a dose level of 4.0 mg/mL was considered to be the MTD. This level was therefore selected for dosing in the 30 d experiment (Experiment 5), and 2.5 mg CAPS/mL was chosen as an intermediate dose level.

Although reticulocytopenia was present in Experiment 4 at 4.0 mg CAPS/mL and above, this change was associated with decreased water consumption, decreased body weight and elevated red cell counts. These associations suggested that the process underlying the inhibition of erythropoiesis was not a direct toxic effect of CAPS. The reduction in water consumption at 5.0 and 6.0 mg CAPS/mL in Experiment 4 (Fig. 4.1) was such that the overall CAPS intake at these levels was, in any case, less than for the animals given 4.0 mg/mL (Table 4.1).

CAPS and CAPS solutions are extremely bitter, and even intravenous administration of CAPS solutions may cause patients to experience a bitter taste (Parfitt, 1999), therefore it is not surprising that mice should find CAPS unpalatable and drink less water. The resulting haemoconcentration in turn may have induced reticulocytopenia by the downregulation of EPO levels. However, increased haematocrit with erythroid suppression is seen within 24 h of the initiation of dehydration in mice, without demonstrable changes in EPO levels (Dunn and Smith, 1980), suggesting that the role of EPO may only be of secondary importance to reduced food intake in producing reticulocytopenia in mice (Dunn, 1980).

Reduction of water intake in rodents is associated with reduced diet intake, and CAP itself has been reported to cause inappetance in laboratory animals (Gruhzit *et al.*, 1949; Reutner *et al.*, 1955; Penny *et al.*, 1967). In the present study, body weight loss was seen at the 5.0 and 6.0 mg CAPS/mL levels, and this was presumably due to reduced diet intake, although this was not measured. It is recognised that reduced food consumption in rodents leads to marrow atrophy and reticulocytopenia (Schwartz *et al.*, 1973; Zaporowska and Wasilewski, 1991; Levin *et al.*, 1993; Matsuzawa and Sakazume, 1994).

In the 30 d study with CAPS administered at 4.0 mg/mL (Experiment 5), haemoconcentration and reticulocytopenia were seen at d 5 (Fig. 4.8). By d 11, RBC was normal and reticulocytes were returning to normal. However, reticulocyte counts continued to rise above control levels until d 15 and were normal thereafter. Body weights, which were initially depressed at the 4.0 mg/mL dose level, were similar to the control value by d 11 (Fig. 4.5). However, water consumption remained depressed to the same extent throughout the experiment (Fig. 4.4). Since body weight, erythrocyte and

reticulocyte counts had begun to normalise by d 11 (Fig. 4.8), it would appear that homeostatic mechanisms were simultaneously responsible for normalising fluid balance, food utilisation and erythropoietic activity. These findings indicated that in spite of reduced water intake at 4.0 mg CAPS/mL, this dose level would be appropriate and would not cause undue toxicity when used in subsequent longer experiments in the present project.

CHAPTER 5: EVALUATION OF BUSULPHAN AND CHLORAMPHENICOL SUCCINATE IN THE INDUCTION OF APLASTIC ANAEMIA IN THE B6C3F₁ MOUSE

5.1 Introduction

It was now intended to study the combination of BU treatment with the subsequent administration of CAPS in the induction of AA. By using a BU dosing regime of four fortnightly doses in B6C3F₁ mice, it was intended to investigate the Type II (Type B) (irreversible) haemotoxicity of CAP (see Section 1.3.2) described by Yunis and Bloomberg (1964) and Chaplin (1986b). If Type II/B haemotoxicity were to develop, such differential toxicity between mice treated with BU alone and mice treated with BU and CAPS might form the basis for a model of drug-induced AA in the mouse.

The experimental design described by Morley *et al.* (1976b), to investigate the haemotoxicity of CAP, employed a treatment regime developed earlier (Morley and Blake, 1974a) in which 10-15 week old female BALB/c mice were given BU ip on four occasions at 20, 20, 20 and 10 mg/kg at intervals of two weeks. This regime had been shown to induce residual marrow damage in animals surviving for two months or more after the final BU administration, and was characterised by near normal peripheral blood cell counts. Forty-one percent of animals had completely normal peripheral blood counts but 65 % of mice showed reduced tibial marrow cellularity (Morley and Blake, 1974a). Following a rest period of six weeks after BU dosing, CAPS was given in the drinking water at 5 mg/mL, then, at various points up to 139 days after the start of CAPS treatment, tibial cellularity, CFU-S and CFU-C (CFU-GM) were measured. Whilst tibial cellularity in mice given BU and CAPS was not significantly different from BU-treated

controls that bore residual marrow damage, CFU-S and CFU-C were depressed on a number of occasions, particularly at later timepoints (Morley *et al.*, 1976b).

The principal aim of the present study was to induce AA in the B6C3F₁ mouse. Animals would be treated with BU at 40 mg/kg, the dose level determined in Experiments 1-3 (Chapter 3), allowed to recover from any acute toxicity for six weeks, then be given CAPS in the drinking water *ad libitum* at 4.0 mg/mL, as determined by Experiments 5 and 6 (Chapter 4). Evidence for CAP Type II/B haemotoxicity would be either excess mortality from AA, or clear differences in the haematology and cellular composition of haemopoietic organs in BU/CAPS-treated animals when compared with those receiving BU alone. Histological examination of a limited range of tissues at later timepoints in the experiment was intended to further characterise the extent of late BU/CAPS toxicity on other organs, especially the eye and lung, and to provide morphological evidence of the haematological lesions in the bone marrow, spleen and other organs of the reticuloendothelial system.

5.2 Materials and Methods

Mice were obtained from the Small Animal Breeding Unit, Glaxo Wellcome Research and Development, Ware, Herts. BU, and CAPS administration in drinking water were as described in Chapter 2. Blood sampling and analysis was as described in Chapter 2, except on d 177 and 210, when 0.2 mL samples were taken by caudal venepuncture. Bone marrow and spleen sampling and analysis were as described in Chapter 2. Lymphocyte subset analysis was performed using Method 1 (Chapter 2) on d 384 and Method 2 on d 485/497. Histopathology was performed as described in Chapter 2. CAP and CAPS analysis was performed using Method 1 (Chapter 2).

5.2.1

Experiment 6: The Administration of Busulphan and Chloramphenicol Succinate to B6C3F₁ Mice: a 497-day Study

Three hundred and thirty-six mice were randomly assigned to 6 groups of 36 (groups 1 to 6) and 6 groups of 20 (groups 7 to 12) (Table 5.1).

Table 5.1: Design of Experiment 6.

Group	n	BU dose, mg/kg	Dosing, d		Sampling ¹ , d									
			1, 16, 30, 43	85 onwards	1	49	85	140	177	210	253	384	485	497
1	36	0	BU	CAPS	B, M	B, M	B, M, H	B	B	B	B	B, L, M, H	B, L	
2	36	10	BU	CAPS		B, M	B, M, H	B	B	B	B	B, L, M, H	B, L	
3	36	18	BU	CAPS		B, M	B, M, H	B	B	B	B	B, L, M, H	B, L	
4	36	25	BU	CAPS		B, M	B, M, H	B	B	B	B	B, L, M, H	B, L	
5	36	33	BU	CAPS		B, M	B, M	B	B	B	B	B, L, M, H	B, L	
6	36	40	BU	CAPS		B, M	B, M	B	B	B	B	B, L, M, H	B, L	
7	20	0	BU					B	B	B	B		B, L	
8	20	10	BU					B	B	B	B		B, L	
9	20	18	BU					B	B	B	B		B, L	
10	20	25	BU					B	B	B	B		B, L	
11	20	33	BU					B	B	B	B		B, L	
12	20	40	BU					B	B	B	B		B, L	

¹Blood counts (B), lymphocyte analysis (L), marrow counts (M), histology (H)

Animals in groups 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12 were dosed on four fortnightly occasions (d 1, 16, 30, and 43) with BU at 0 (controls), 10, 18, 25, 33 and 40 mg/kg, respectively. Six weeks after the fourth dose of BU, on d 85, animals in groups 1 to 6 were given 4.0 mg CAPS/mL in the drinking water *ad libitum* whilst those in groups 7 to 12 were given drinking water without the antibiotic. Five animals per group were killed at various time points and blood samples were taken for full blood count, differential leucocyte count and reticulocyte count, and bone marrow and spleens for nucleated cell counts. On d 1 group 1 animals only were sampled. On d 49 (6 d after the last BU dose), d 85 (the first d of CAPS administration) and d 384, samples were taken from groups 1-6 only. On five occasions, d 140, 177, 210, 253 and 485/497, samples were taken from all twelve groups. For logistical reasons, groups 1 to 6 were bled for the last time on d 485, and groups 7 to 12 on d 497. On d 177 and 210, 0.2mL blood samples

were taken by caudal venepuncture only; bone marrow and spleen samples were therefore not taken on these two occasions. Lymphocyte subset analysis was performed on d 384 and 485/497. For the determination of serum levels of CAP, blood was taken from 5 animals from groups 1 to 7 on d 485. CAPS concentration analysis was performed on CAPS stock solutions and water bottle dosing solutions after the end of the experiment on d 497. Water consumption of all groups was recorded twice weekly, and body weights weekly. Histological examination was performed on 5 animals per group in groups 1 to 6 on d 384. In addition, tissues were taken for histology from 5 animals per group in groups 1-4 on d 85 to assess BU-induced changes prior to CAPS administration, but not from groups 5 and 6 (to allow for possible excess mortality in these two high-dose BU groups). Tissues were also taken from seven animals which died unexpectedly between d 107 and 217.

5.3 Results

5.3.1 Mortality, Clinical Signs and Haematology of Intercurrent Death Animals

During the experiment, a number of mice were killed when their condition deteriorated, and others were found dead; these animals are referred to as intercurrent deaths (ICDs). ICD animals, together with selected haematological values, are listed in Table 5.2. This table is subdivided into four chronological sections, (a) and (b), mice with marrow aplasia; (c) and (d), mice with evidence of lymphoma. Sections (b) and (c) consist of animals which received a limited autopsy. A further section, (e), shows details of mice with no evidence of aplasia or lymphoma.

Table 5.2: Individual haematological values of ICD mice treated with BU on d 1, 16, 30 and 43. Where no value is shown, appropriate samples were not available, or were insufficient for analysis. fd = found dead; a = limited autopsy performed. Femoral infiltration: + = marrow replaced with lymphoma cells; 0 = no infiltration; (+) = few or occasional lymphoma cell(s) present. The table is subdivided into 5 sections: a and b, mice with marrow aplasia; c and d, mice with evidence of lymphoma; e, mice with no evidence of aplasia or lymphoma. Sections b and c consist of animals which received a limited autopsy.

Animal no.	BU dose level (mg/kg)	Day	Femur TNC ($\times 10^7$)	Femoral infiltration	Spleen TNC ($\times 10^7$)	Hb (g/dL)	Retic ($\times 10^9/L$)	PLT ($\times 10^9/L$)	WBC ($\times 10^9/L$)	Neut ($\times 10^9/L$)	Lymph ($\times 10^9/L$)	LUC ($\times 10^9/L$)
(a): d 17 to 62. Mice with marrow aplasia												
1	33	17										
2 fd	33	18										
3 fd	33	18										
4 fd	40	18										
5 fd	40	18										
6 fd	40	18										
7	40	20	0.3	0	6.2	15.2	9.5	736	4.0	0.44	3.40	0.07
8 fd	40	20										
9 fd	33	29										
10	40	30	0.4	0	11.4							
11	33	31	0.4	0	8.3							
12 fd	33	31										
13 fd	40	31										
14 fd	33	46										
15	33	48	0.4	0	2.5							
16 fd	40	48		0	14.5							
17 fd	40	50	1.1	0								
18	40	51	0.5	0	10.1	1.9	19	1.1	0.02	0.91	0.13	
19	40	62	0.6	0	6.8	2.3	11.6	11	1.7	0.03	1.39	0.23
(b): d 107 to 168. Mice with marrow aplasia, limited autopsy performed												
20 a	40 + CAPS	107	<0.1	0	4.8	1.9	1.2	66	1.1	0.10	0.97	0.02
21 fd	40	118	<0.1	0								
22 a	40	129	0.6	0	4.8	2.1	18.0	41	1.3	0.18	0.93	0.20
23 a	25 + CAPS	168	1.3	0	1.5	12.4		816	1.5	0.92	0.62	

Table 5.2 (continued)

Animal no.	BU dose level (mg/kg)	Day	Femur TNC ($\times 10^7$)	Femoral infiltration	Spleen TNC ($\times 10^7$)	Hb (g/dL)	Retic ($\times 10^9/L$)	PLT ($\times 10^9/L$)	WBC ($\times 10^9/L$)	Neut ($\times 10^9/L$)	Lymph ($\times 10^9/L$)	LUC ($\times 10^9/L$)
(c): d 174 to 217. Mice with evidence of lymphoma, limited autopsy performed												
24 fd	33 + CAPS	174		+								
25 a	40 + CAPS	175	0.3	+	23.4	2.8	378.0	61	3.8	0.15	3.48	
26 a	40	184	2.8	+	78.8	12.5	90.0	119	16.5	0.22	6.35	9.62
27 a	40	191	2.2	+	123.4	15.7	262.0	234	132.0	2.02	67.24	51.08
28 fd,a	40	217		+								
(d): d 237 to 437. Mice with evidence of lymphoma												
29	40 + CAPS	237		+	19.2	15.7	476.3	506	3.2	1.19	1.55	0.37
30	40	244	3.0	(+)	27.4	17.2	224.0	372	14.2	2.72	5.90	4.35
31	33	252	3.5	+	123.0	12.7	237.0	40	81.7	0.85	45.92	34.23
32	33 + CAPS	254		+	1660.0	11.6		178	6.7	1.04	2.32	3.22
33	40 + CAPS	265	2.1	+	136.4	4.5	64.7	138	45.7	1.33	6.44	7.79
34	40	265	1.7	+	66.2	10.0	119.7	90	18.9	0.25	4.82	13.80
35	25 + CAPS	274	1.9	+	81.0	11.7	39.6	199	216.0	4.59	136.83	72.36
36	40	279	1.7	+	63.3	14.2	243.5	186	71.6	2.70	35.23	31.93
37	40	306	4.3	+	91.2	11.8	491.3	117	86.1	6.32	34.09	44.34
38	33 + CAPS	328	3.3	(+)	32.0	15.1	81.9	264	4.9	1.30	2.46	0.86
39	40 + CAPS	371	1.8	+	46.0	14.2	683.5	55	18.7	0.78	5.07	12.81
40	40 + CAPS	380	1.5	+	107.5	12.8	744.2	40	188.1	1.71	92.61	93.11
41	10 + CAPS	398	3.9	+	89.8	12.6	339.8	305	16.5	0.63	11.50	3.58
42	0	416	3.2	(+)	23.4	14.1	88.2	1220	12.4	2.31	8.67	0.53
43 fd	40 + CAPS	418	1.5	+	34.7							
44	10	437		+		18.4	84.0	186	2.6	1.19	1.12	0.07
(e): d 185 to 465. Mice with no evidence of marrow aplasia or lymphoma.												
45 fd	25	185	4.3	0	13.3							
46	18 + CAPS	355	3.3	0		14.8	222.9	854	9.8	2.24	6.47	0.18
47	40	359	3.3	0	43.4	9.9	604.3	1442	29.8	21.15	5.21	2.50
48	40 + CAPS	465	1.7	0	9.9	14.3	213.7	549	3.7	0.89	2.42	0.02

Early ICDs occurred in the period immediately following each BU administration. For example, after the second dose of BU on d 16, one ICD mouse was recorded on d 17, 5 on d 18, and 2 on d 20 (Table 5.2 (a)). Of 17 animals dying up to d 50 (Table 5.2 (a)), all had received 33 or 40 mg BU/kg, and all but five (no. 1, 7, 10, 11 and 15) were found dead at the morning inspection. A further 31 animals died between d 51 and the end of the experiment (Table 5.2 (a)-(e)). Five of these were found dead (no. 21, 24, 28, 43 and 45), but the remaining 26 tended to show marked weight loss and loss of condition, and were sacrificed and blood, bone marrow and/or spleen samples were taken.

Where samples were available for counting between d 17 and 62 (Table 5.2 (a), no. 7, 10, 11, 15, 17, 18 and 19), bone marrow hypocellularity was present in six of the seven animals. Marked reticulocytopenia was evident in two of the three peripheral blood samples available (Table 5.2 (a), no. 7 and 19) and assessment of polychromasia in the smear of the third (Table 5.2 (a), no. 18) suggested a failure to compensate the severe anaemia in this animal.

Six animals died between d 51 and d 168 (Table 5.2 (a) and (b), no. 18 to 23). One (no. 21) was found dead, but the other five were sacrificed, of which three (Table 5.2 (b), no. 20, 22 and 23) were given a limited autopsy. Five (no. 18 to 22) of the six animals had received 40 mg BU/kg, and showed moderate or marked marrow hypocellularity or cytopenias (Fig. 5.1; 5.2 (a)). The other mouse had been dosed at 25 mg/kg (Table 5.2 (b), no. 23). Femoral cellularity and platelet count were normal in this animal, but there was loss of haemopoietic tissue in the sternum, and splenic cellularity and lymphocyte count were markedly reduced.

From d 174 to the end of the experiment, 25 animals died (Table 5.2 (c), (d) and (e). In twenty-one there was evidence of lymphoma development (Table 5.2 (c) and (d)).

Infiltration of the femoral marrow cavity by lymphoma cells, splenomegaly or the presence of lymphoma cells in peripheral blood smears, was seen in all 21 animals (Fig. 5.2 (b)). Morphologically, the lymphoma cell seen in smears and spleen imprints was a large oval cell with intense cytoplasmic basophilia, high nuclear/cytoplasmic ratio, a relatively immature nucleus and occasional cytoplasmic vacuolation (Fig. 5.3). α -naphthyl acetate esterase and acid phosphatase activity were seen as a single, small localised area of the cytoplasm, as distinct from an overall, diffuse pattern (Fig. 5.3). All but one of the 25 lymphoma animals (Table 5.2 (d), no. 42) had received BU.

Of the four animals dying between d 185 to 465 with no evidence of aplasia or lymphoma (Table 5.2 (e)), one (no. 45) was found dead unexpectedly; another (no. 46) presented with weight loss, the third (no. 47) had an infected subcutaneous mass and the fourth (no. 48) a probable middle ear infection.

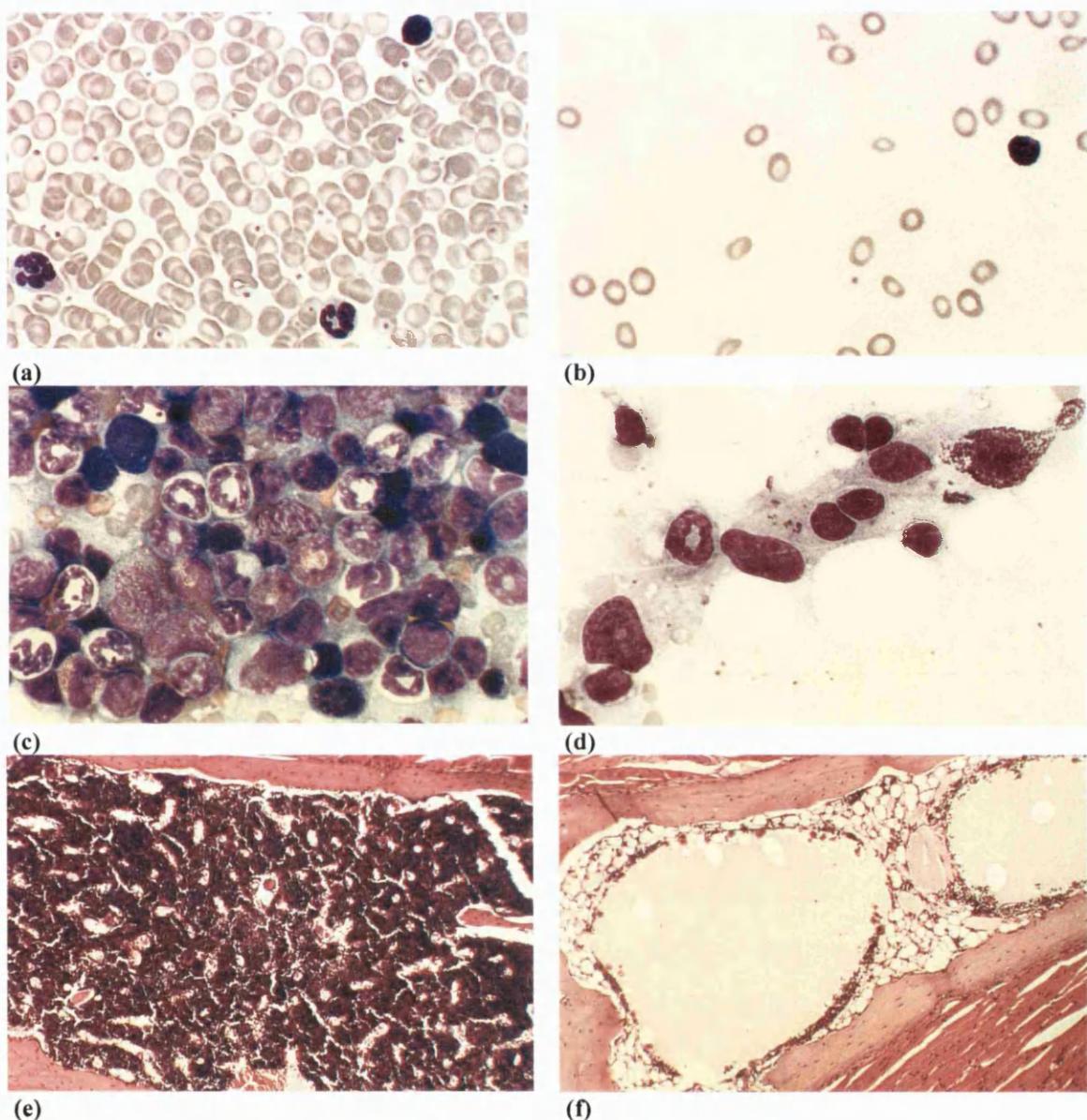


Figure 5.1: Representative peripheral blood and bone marrow morphology in BU-treated aplastic ICD mice dying up to d 168 in Experiment 6.

- (a) Control, d 85: peripheral blood smear, MGG $\times 900$. Hb = 16.0 g/dL; WBC = $7.0 \times 10^9/L$; Plt = $1008 \times 10^9/L$.
- (b) ICD, d 107 (Table 5.2 (b), no. 20): peripheral blood smear, MGG $\times 900$; Hb = 1.9 g/dL, WBC = $1.1 \times 10^9/L$; Plt = $66 \times 10^9/L$. Erythrocytes show moderate anisocytosis and poikilocytosis.
- (c) Control, d 85: femoral marrow smear, MGG $\times 900$. TNC = 2.96×10^7 . There is a normal distribution of cells of the myeloid, erythroid and lymphoid lineages.
- (d) ICD, d 107: femoral marrow smear, MGG $\times 900$. TNC = $<0.1 \times 10^7$. Erythroid and early myeloid precursors are absent. The only other remaining cells are lymphocytes, macrophages and mast cells.
- (e) Control, d 85: section of sternal bone marrow, H&E $\times 90$. The marrow cavity is fully populated with haemopoietic cells; megakaryocytes are clearly visible.
- (f) ICD, d 107: section of sternal bone marrow, H&E $\times 90$. There is marked hypoplasia with fatty replacement. Two large cystic areas are seen.

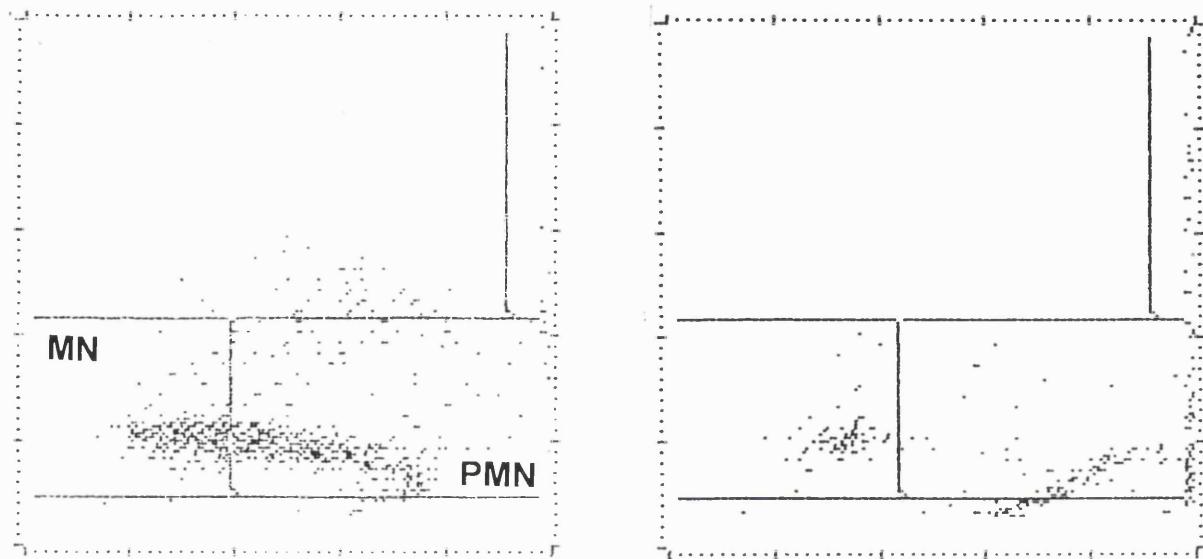


Figure 5.2 (a): Bayer H*1 cytograms.

Femoral nucleated cells in basophil channel. Vertical axis = forward light scatter (size); horizontal axis = high angle light scatter (nuclear density). The lower vertical threshold separates mononuclear (MN) from polymorphonuclear (PMN) cell nuclei

L: control mouse, d 85, nucleated cell count 3.6×10^7 .

R: hypoplastic mouse (Table 5.2 (b), no. 22, d 129), nucleated cell count 0.6×10^7 . There is a marked depletion, in particular, of polymorphonuclear cells. Fat particles are seen as a sigmoid tail in the lower right area and extending into the upper right area.

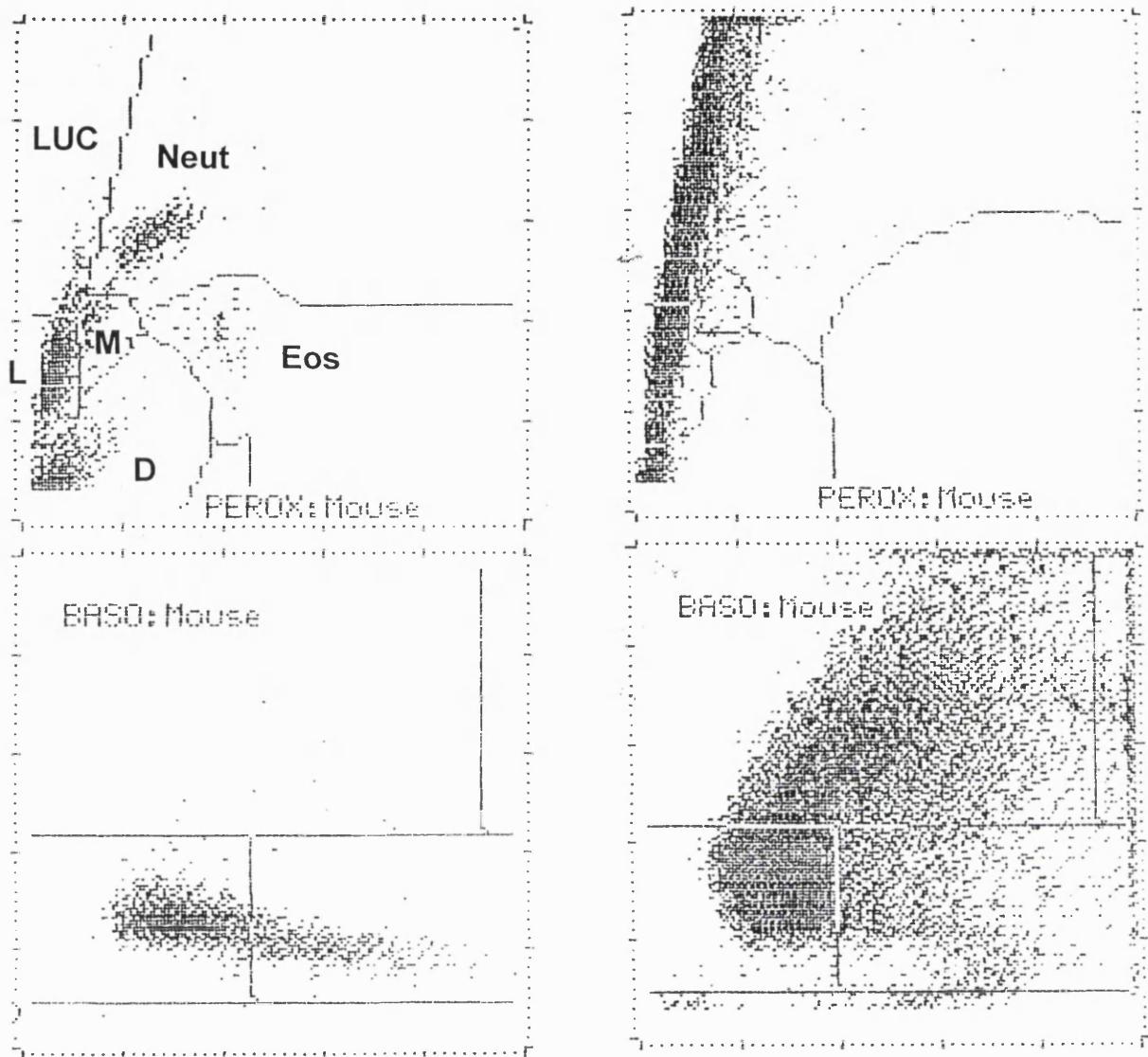


Figure 5.2 (b): Bayer H*1 cytograms.

Peripheral blood in peroxidase (PEROX) and basophil (BASO) channels. PEROX vertical axis = forward light scatter (size); horizontal axis = absorption (staining intensity). L, lymphocytes; M, monocytes; D, debris and platelet aggregates.

L: control, d 384. WBC $7.1 \times 10^9/L$, lymphocytes $5.48 \times 10^9/L$ (77.1 %), LUC $0.27 \times 10^9/L$ (3.8 %); R: mouse with lymphoma (Table 5.2 (d), no. 40, d 380). WBC $188.1 \times 10^9/L$, lymphocytes $92.61 \times 10^9/L$ (49.2 %), LUC $93.04 \times 10^9/L$ (49.5 %). In the peroxidase channel cytogram, the marked increase in lymphocytes and LUC, and depletion of granulocytes, is clearly seen. In the basophil channel cytogram, the majority of cells are counted as mononuclear nuclei, however, a substantial proportion appear resistant to lysis (falling above the upper horizontal threshold) and are classified as basophils by the analyser.

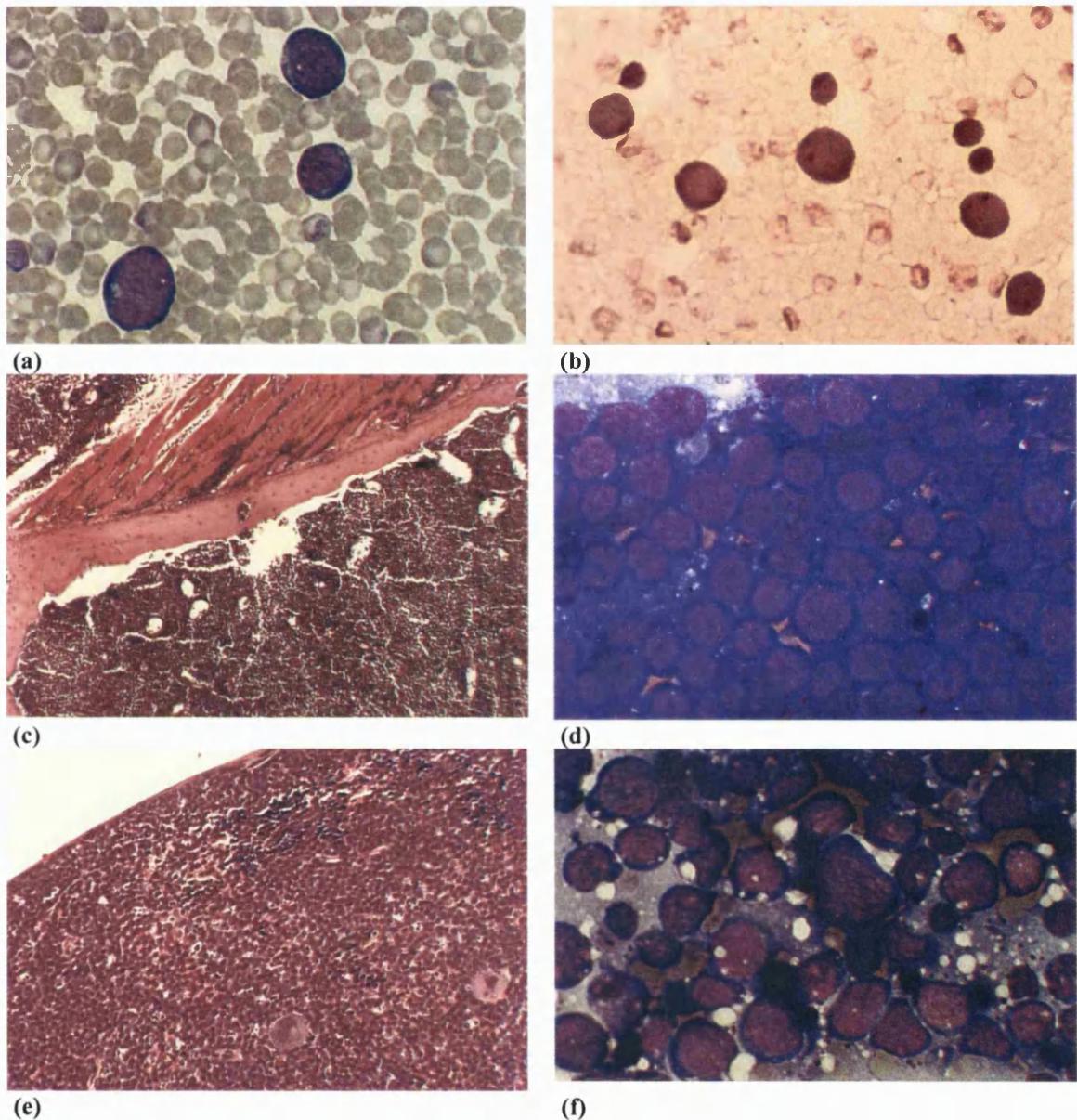


Figure 5.3: Representative morphological features in peripheral blood, bone marrow and spleen in lymphoma bearing ICD mice dying after d 168 in Experiment 6. (a), (b), (d) and (f), no. 39, d 371 (Table 5.2(c)). (c) and (e), no. 26, d 184 (Table 5.2(c)).

- (a) Peripheral blood smear, MGG $\times 900$. WBC = $18.7 \times 10^9/L$, LUC = $12.81 \times 10^9/L$. Five large, oval lymphoma cells are seen. The cells have a high nuclear:cytoplasmic ratio, the nuclei are immature and nucleoli are present. The cytoplasm is deeply basophilic and the occasional vacuole can be seen.
- (b) Peripheral blood smear, acid phosphatase and haematoxylin $\times 900$. The localised acid phosphatase positivity (red) in the lymphoma cells is mostly confined to a single dot.
- (c) Section of sternum, H&E $\times 360$. There is complete replacement of the marrow cavity and infiltration of the adjacent intercostal muscles by lymphoma cells.
- (d) Femoral smear, MGG $\times 900$. Uniform sheets of lymphoma cells are present.
- (e) Section of spleen, H&E $\times 90$. The normal architecture is largely effaced by infiltrating lymphoma cells.
- (f) Spleen imprint, MGG $\times 900$. The majority of cells visible are infiltrating lymphoma cells, however, a number of normal lymphoid cells can be seen, together with some erythroid precursors.

5.3.1.2 Influence of Chloramphenicol Succinate on Mortality

Of four animals presenting with aplasia after the start of CAPS dosing on d 85 (Table 5.2 (b), no. 20 to 23, days 107 to 168), all four of which had been treated with BU, two had also received CAPS (no. 20 and 23) and two had not (no. 21 and 22).

Of the 21 animals with evidence of lymphoma between d 174 and the end of the experiment (Table 5.2 (c) and (d)), all but one (no. 42) had previously received BU.

Eleven of these 21 mice had received CAPS and ten had not (Table 5.3). The mean and median day of occurrence of lymphoma ICDs were similar in animals receiving CAPS and in those that did not receive CAPS. Analysis of the numbers, and of the time of occurrence, of ICDs in respect of CAPS administration showed that CAPS had no effect on lymphoma death (hazard ratio = 0.98; 95 % confidence intervals 0.42 - 2.31 after adjusting for BU dose).

Table 5.3: The influence of CAPS administration on the occurrence of ICDs due to lymphoma occurring after d 168 (the day of the last aplastic case).

BU dose level (mg/kg)	With CAPS			Without CAPS		
	Group	Days of ICD	Total	Group	Days of ICD	Total
0	1			7	416	1
10	2	398	1	8	437	1
18	3			9		
25	4	274	1	10		
33	5	174, 254, 328	3	11	252	1
40	6	175, 237, 265, 371, 380, 418	6	12	184, 191, 217, 244, 265, 279, 306	7
Total			11			10
Mean d of ICD (sd)		297.6 (86.7)		279.1 (86.4)		
Median d of ICD		274		258.5		

5.3.1.3 Influence of Busulphan on Mortality

In view of the fact that mortality occurred with almost equal frequency in groups receiving CAPS and those that did not receive the drug, an analysis was performed on the effect of BU dosing, ignoring CAPS treatment, on ICD frequency and time to ICD occurrence. This is presented graphically in Fig. 5.4, and shows a clear dose-dependent effect; a 10 mg/kg increase in dose giving a hazard ratio of 2.23 (confidence intervals 1.42 - 3.52). Survival in animals dosed at 40 mg BU/kg was 41.3 % at the end of the experiment. In the 33 mg and 25 BU/kg groups, this figure was 73.9 % and 93.5 %, respectively.

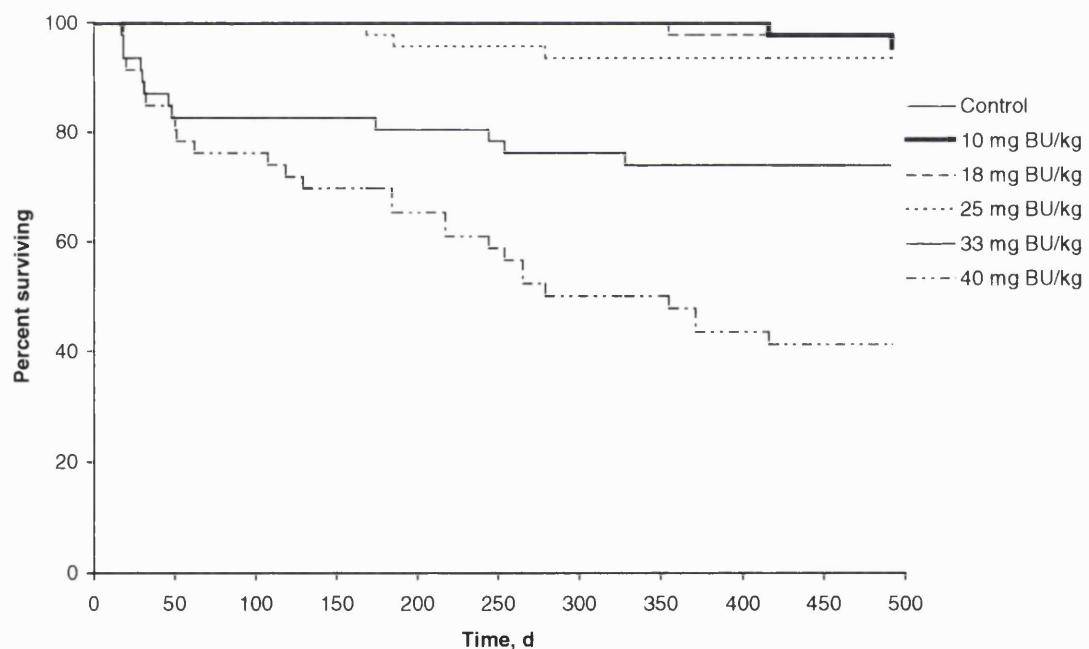


Figure 5.4: Survival of mice dosed with BU (see key for dose level). Survival is expressed as the percentage of the combined original group sizes (i.e., BU alone group and BU + CAPS group; n=56) at each dose level. There is a clear dose-dependent effect. Note that the plot of the control group is obscured by that of the 10 mg BU/kg group.

Other animals appeared well throughout the remainder of the experiment. No effect was seen on the mean body weights of animals in any dose group. However, in mice receiving 33 and 40 mg BU/kg, fur greying was apparent in particular animals from about d 96 (Fig. 5.5), and by the end of the study, most animals in these two treatment groups were affected in this way.



(a)



(b)

Figure 5.5: Fur greying in BU treated mice. (a) and (b) Mice on d 321 from a single cage of animals receiving 40 mg BU/kg. Note also in (b) the animal (right) with marked hair loss.

5.3.2 Haematology

On the five occasions when blood samples were taken from all dose groups, that is, d 140, 177, 210, 253 and 485/497 (Table 5.1), four separate comparisons were made of the haematological results and these were statistically analysed: an example of these comparisons is shown in Table 5.4, where platelet counts on d 140 are analysed. The four statistical comparisons were: (a) the effect of BU + CAPS versus BU vehicle/CAPS (groups 2 - 6 vs. group 1); (b) the effect of BU alone versus BU vehicle alone (groups 8 - 12 vs. group 7); (c) the effect of BU + CAPS versus BU vehicle alone (groups 2 - 6 vs. group 7) and (d) the effect of BU + CAPS versus BU alone (pairwise comparison of groups 1 vs. 7, 2 vs. 8, 3 vs. 9, 4 vs. 10, 5 vs. 11 and 6 vs. 12). Representative plots, showing analysis (a) and (b), of haemoglobin, reticulocyte counts, platelet counts and lymphocyte counts are shown in Fig. 5.6. Analysis (a) (the effect of BU + CAPS versus BU vehicle/CAPS) and analysis (b) (the effect of BU alone versus BU vehicle alone) demonstrate the effects of BU treatment on some parameters which are described below (Sections 5.3.2.1 to 5.3.2.3). In general, analysis (c) produced significance levels that were very similar to analyses (a) and (b).

Analysis (d), the pairwise comparison of each BU + CAPS dose group against the corresponding BU-only dose group, on the other hand, gave results that were very often non-significant (for example, Table 5.4, for analysis of platelet counts on d 140); statistically significant differences which did emerge in other haematological parameters at other times were relatively small and inconsistent.

Table 5.4: Statistical analysis of haematological parameters in Experiment 6. The analysis of platelet counts on d 140 is shown below as a representative example. This particular data is also shown graphically in Fig. 5.6 (c). n=5, except that values were not obtained for mice sampled in group 2 due to the presence of platelet aggregates in the samples.

Group	BU dose, mg/kg	CAPS, 4.0 mg/mL	mean, $\times 10^9/L$	sd, $\times 10^9/L$	analysis (t test)	
					vs group 1 (a)	vs group 7 (c)
1	0	YES	1178.8	36.0	-	-
2	10	YES	-	-	-	-
3	18	YES	982.5	62.9	0.003	0.12
4	25	YES	774.8	141.7	0.0003	0.003
5	33	YES	915.8	36.5	0.00001	0.01
6	40	YES	779.4	141.9	0.0003	0.003
Group	BU dose, mg/kg	CAPS, 4.0 mg/mL	mean $\times 10^9/L$	sd $\times 10^9/L$	vs group 7 (b)	pairwise (d)
7	0	NO	1233.8	162.9	-	1 vs 7
8	10	NO	996.8	130.4	0.05	2 vs 8
9	18	NO	933.3	146.5	0.03	3 vs 9
10	25	NO	772.4	260.1	0.02	4 vs 10
11	33	NO	900.7	149.9	0.04	5 vs 11
12	40	NO	799.8	150.9	0.004	6 vs 12

This detailed analysis between the various treatment groups indicated that the

administration of CAPS had no influence on any haematological parameter.

Therefore data from groups 1 to 6 and from groups 7 to 12, regardless of CAPS

administration, was pooled for subsequent analysis (i.e., group 1 data was combined with group 7 data, group 2 with group 8, group 3 with group 9, group 4 with group 10, group 5 with group 11 and group 6 with group 12; Table 5.5).

Table 5.5: Combined platelet counts from mice sampled on d 140 and statistical analysis for BU treatment. These data are shown graphically as part of Fig. 5.6 (c). Data shown for groups 2 and 8 was from group 8 only. n=10

Combined groups	BU dose, mg/kg	mean $\times 10^9/L$	sd $\times 10^9/L$	t test: p vs combined groups 1 and 7
1 and 7	0	1203.2	107.0	-
2 and 8	10	996.8	130.4	0.008
3 and 9	18	949.7	119.6	0.0009
4 and 10	25	773.6	197.5	0.00002
5 and 11	33	909.3	90.7	0.00004
6 and 12	40	789.6	138.5	0.000001

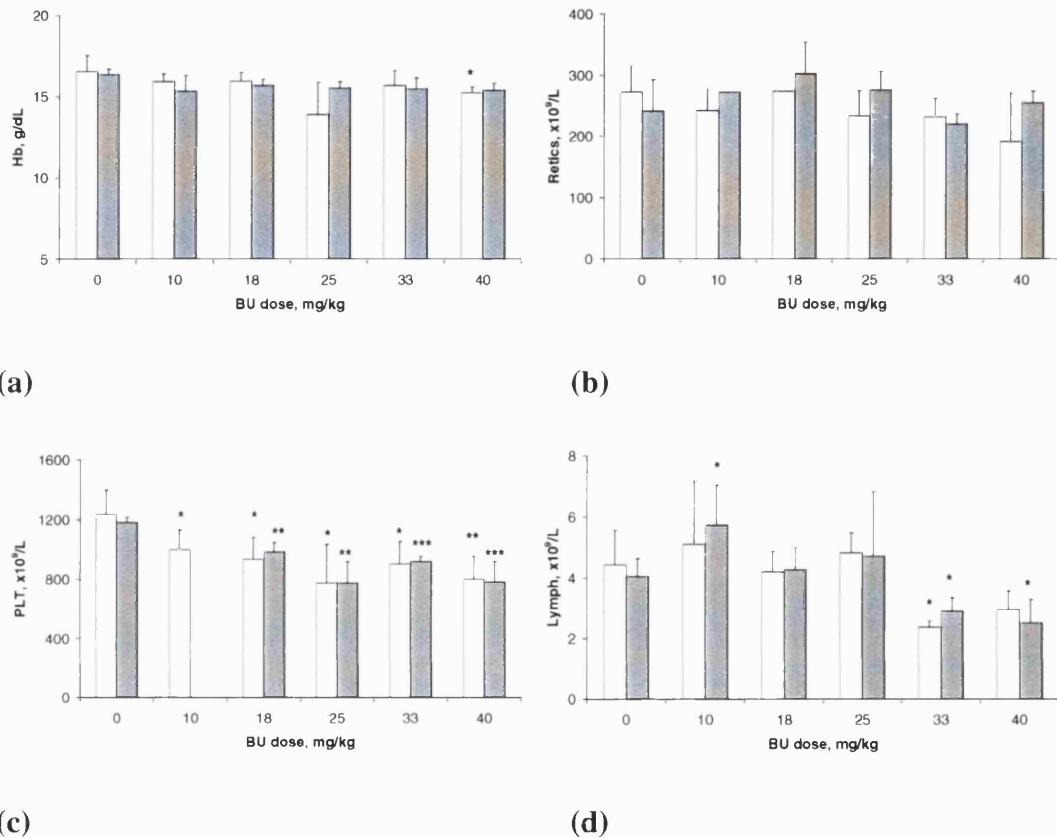


Figure 5.6: Representative plots of haemoglobin and peripheral cell counts in mice treated with BU. Means and sd of (a) haemoglobin concentration, d 253; (b) reticulocyte count, d 177; (c) platelet count, d 140; (d) lymphocyte count, d 210. Empty bars, BU alone; shaded bars, BU + CAPS. *p<0.05; ** p <0.01; * p <0.001: significance of effect of BU treatment. n=5, except for (c) group 2 (BU + CAPS) at d 140 where platelet aggregates were present. Comparisons of BU treatment groups were made as follows: groups 8 to 12 (mice given BU alone) versus group 7 (BU vehicle alone); groups 2 to 6 (mice given BU + CAPS) vs. group 1 (BU vehicle + CAPS alone); see Table 5.4.**

5.3.2.1 **Bone Marrow and Spleen**

Total bone marrow counts in control animals receiving CAPS alone (group 1) showed an increase over the course of the experiment as would be expected in growing animals (Table 5.6), increasing from 1.97×10^7 at d 1 after the commencement of BU dosing, to 1.88, 2.52 and 3.56×10^7 at d 49, 85 and 384, respectively. Similarly, since the proportions of myeloid, erythroid and lymphoid cells remained relatively constant at all times in these control animals, the total numbers of each of these cell types generally showed an increase with time. Thus, the numbers of myeloid cells in group 1 control animals at d 1, 49, 85 and 384 were 0.82, 0.78, 0.93 and 1.23×10^7 , respectively. For erythroid and lymphoid cells, these values were 0.56, 0.58, 0.73, 1.23×10^7 , and 0.54, 0.47, 0.77, 1.03×10^7 , respectively (Table 5.6).

However, the total bone marrow cell counts in mice which received BU and CAPS (groups 2 to 6) showed no significant effects due to BU administration. In a similar way, the administration of BU at all dose levels did not significantly affect the numbers of myeloid and erythroid cells, although there was some evidence of a trend of reduced numbers of cells at high BU dose levels at particular timepoints (eg erythroid cells at d 384). Also, there was a general trend for reduction of all cell types at 40 mg/kg on d 49, but this was not significant. However, femoral lymphoid cell numbers were increased on d 49 at 10, 18, 25 and 33 mg BU/kg. At 33 mg/kg, this increase was significant. At d 85, there were slight reductions in femoral lymphoid cell numbers in all BU dose groups, but these reductions were not significant. At d 384, femoral lymphoid cell numbers were similar in all dose groups except for a slight, but non-significant reduction in the group receiving 40 mg BU/kg.

Table 5.6: Total femoral nucleated cells, and myeloid, erythroid, and lymphoid cell counts at scheduled kills in mice treated with CAPS alone (group 1) and BU + CAPS (groups 2 to 6; Table 5.1). Figures are mean counts $\times 10^7$ (sd). n=5; *significantly different to control value, p<0.05.

Group	Busulphan dose (mg/kg)	Femoral cells	Day of experiment			
			1	49	85	384
1	0	Total	1.97 (0.39)	1.88 (0.52)	2.52 (0.93)	3.56 (0.59)
2	10			1.89 (0.12)	1.90 (0.63)	3.32 (0.36)
3	18			2.01 (0.48)	2.23 (0.74)	3.68 (0.83)
4	25			2.15 (0.23)	1.56 (0.39)	3.72 (0.99)
5	33			2.30 (0.60)	2.50 (0.44)	3.20 (0.59)
6	40			1.38 (0.69)	2.16 (0.92)	3.46 (0.44)
1	0	Myeloid	0.82 (0.18)	0.78 (0.19)	0.93 (0.32)	1.23 (0.20)
2	10			0.63 (0.19)	0.74 (0.24)	1.19 (0.17)
3	18			0.56 (0.28)	0.91 (0.40)	1.30 (0.33)
4	25			0.63 (0.11)	0.56 (0.11)	1.40 (0.28)
5	33			0.73 (0.33)	1.02 (0.18)	1.31 (0.18)
6	40			0.66 (0.50)	0.89 (0.34)	1.66 (0.60)
1	0	Erythroid	0.56 (0.13)	0.58 (0.25)	0.73 (0.31)	1.23 (0.30)
2	10			0.56 (0.15)	0.61 (0.24)	1.11 (0.17)
3	18			0.71 (0.24)	0.64 (0.24)	1.27 (0.25)
4	25			0.68 (0.03)	0.48 (0.14)	1.30 (0.45)
5	33			0.75 (0.23)	0.86 (0.32)	0.84 (0.25)
6	40			0.45 (0.21)	0.66 (0.25)	0.96 (0.27)
1	0	Lymphoid	0.54 (0.12)	0.47 (0.19)	0.77 (0.39)	1.03 (0.24)
2	10			0.69 (0.17)	0.48 (0.17)	0.95 (0.21)
3	18			0.70 (0.16)	0.62 (0.15)	1.03 (0.24)
4	25			0.78 (0.22)	0.50 (0.21)	0.98 (0.32)
5	33			0.79 (0.09)*	0.53 (0.21)	0.91 (0.25)
6	40			0.24 (0.06)	0.53 (0.33)	0.75 (0.27)

Table 5.7: Total splenic nucleated cell counts at scheduled kills in mice treated with CAPS alone (group 1) and BU + CAPS (groups 2 to 6; Table 5.1). Figures are mean counts $\times 10^7$ (sd). n=5; *significantly different to control value, p<0.05, **p<0.01, *p<0.001.**

Group	Busulphan dose (mg/kg)	Day of experiment			
		1	49	85	384
1	0	16.35 (1.83)	15.81 (1.36)	17.96 (2.79)	29.08 (4.32)
2	10		14.68 (2.94)	13.68 (1.43)*	27.40 (5.15)
3	18		14.26 (1.77)	13.54 (1.42)*	32.30 (9.01)
4	25		12.82 (1.56)**	11.96 (2.79)**	30.93 (9.27)
5	33		12.69 (2.29)**	12.06 (1.63)**	22.75 (3.21)*
6	40		11.57 (0.99)***	12.79 (1.53)**	23.22 (5.28)

Splenic cellularity in control (CAPS-only) animals (group 1), as with femoral cellularity, showed a general increase with time (Table 5.7). This trend was not clearly evident over the period from d 1 to d 85, but at d 384, counts showed a definite increase. Thus, control splenic cell counts were 16.35×10^7 at d 1, and 15.81, 17.97 and 29.08×10^7 at d 49, 85 and 384 respectively. On d 49 and 85, there were reductions in splenic cellularity at all BU dose levels with highly significant reductions at 25, 33 and 40 mg BU/kg. On d 49, the reductions in cellularity were dose-related, but at 10 and 18 mg BU/kg, the reductions did not achieve statistical significance. However, at d 85, the decreases were statistically significant at all BU dose levels. At d 384, there were reductions in splenic cellularity at 33 and 40 mg BU/kg, but only the reduction at 33 mg/kg was significant. At 40 mg BU/kg, the reduction in splenic cellularity was to 73.2 %, 71.2 % and 79.8 % of the control value on d 49, 85 and 384, respectively.

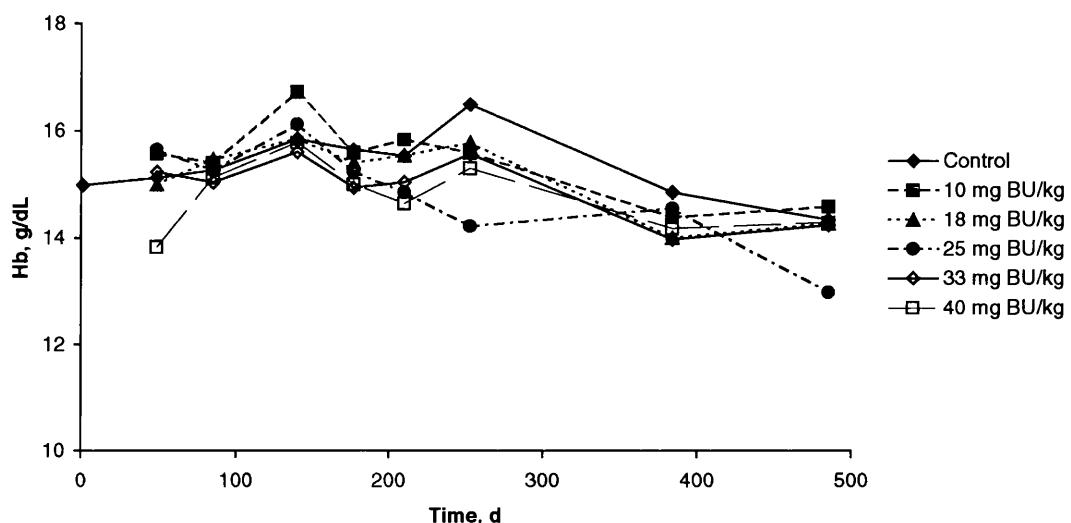
5.3.2.2 Erythrocytes and Platelets

In the analysis of BU treatment groups, pooling data from animals treated with BU alone, and BU + CAPS, erythrocyte count, haemoglobin concentration and haematocrit gradually increased in control animals from d 1 to d 253 and declined thereafter; data for haemoglobin concentration is presented in Fig. 5.7 (a). Reductions in erythrocyte count, haemoglobin concentration, and haematocrit were present in most BU treatment groups, especially from d 177 onwards, but at no time were these reductions significant. These BU-induced decreases were sometimes, but not consistently, accompanied by an increase in MCV. The mean haemoglobin in the 40 mg BU/kg group was depressed at d 49 (Fig. 5.7 (a)), although not significantly, and the mean reticulocyte count and percentage macrocytes in this BU group were both increased at this time. However, only the increase in reticulocyte count achieved statistical significance. The reticulocyte percentage of control mice remained fairly constant throughout the experiment (Fig. 5.7 (b)). In BU-treated mice the reticulocyte counts did not differ from the control values in any consistent manner. However, the mean reticulocyte count was elevated in the 25 mg BU/kg group on d 497 by one individual whose count was $1222.6 \times 10^9/\text{L}$ (56.3 % of RBC); the haemoglobin concentration in this mouse was 3.8 g/dL. The control mean reticulocyte count on this occasion was $300.78 \times 10^9/\text{L}$ (3.13 %), and control mean haemoglobin, 14.34 g/dL.

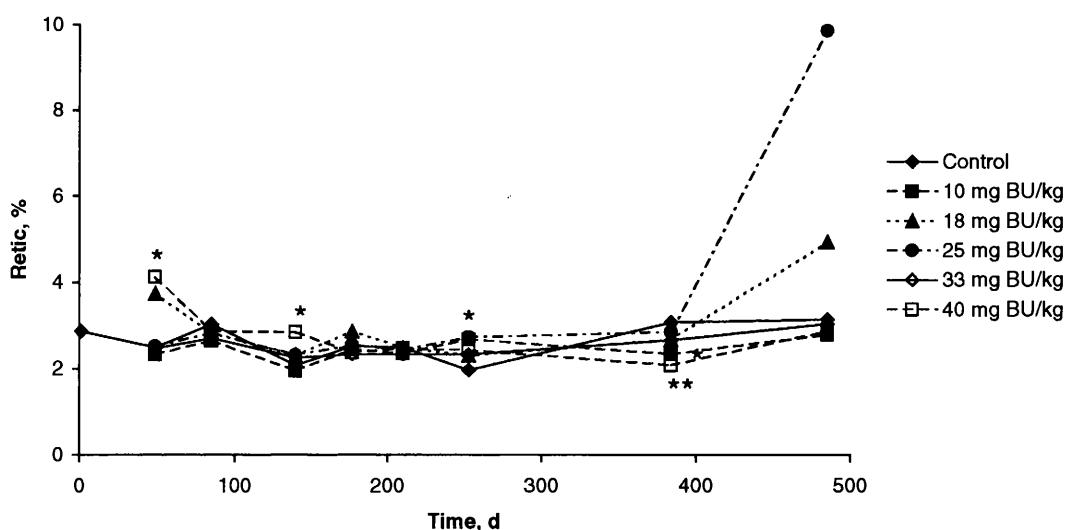
Platelet counts (Fig. 5.7 (c)) showed considerable variability in both control and BU-treated groups. Indeed, standard deviations for control group animals ranged between <40 - $>400 \times 10^9/\text{L}$ on different occasions. This variability was probably attributable to the proficiency of individual operators involved in the blood sampling, and was especially evident at d 177 and 210, and for this reason, counts obtained on these occasions are not shown. Nevertheless, in BU-treated mice, highly significant reductions of counts were

evident on d 49, 85 and 140 in most dose groups, and the decreases were, in general, related to BU dose. However, after d 140, the counts in all treated groups became closer to the control mean.

(a)



(b)



(c)

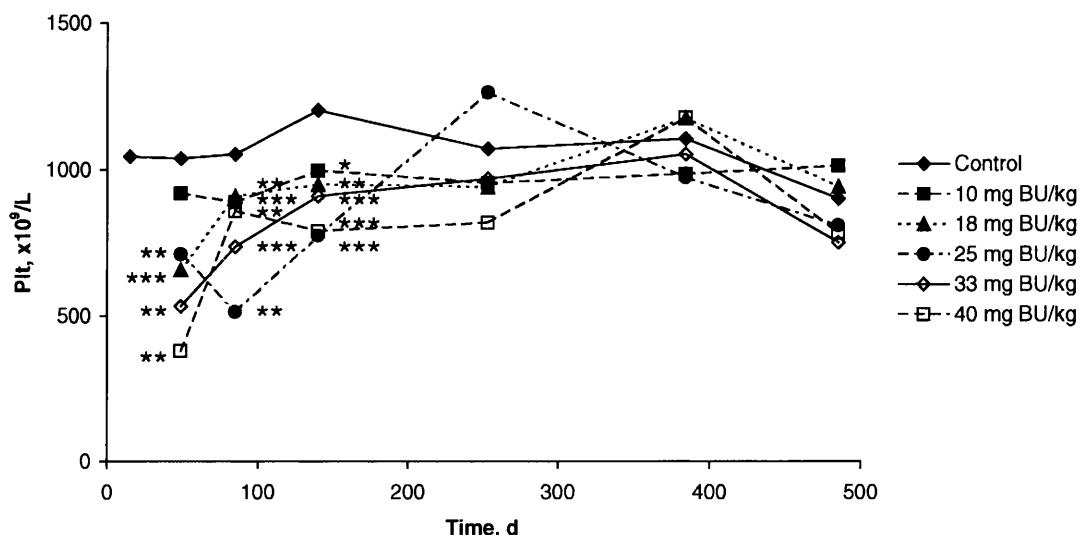


Figure 5.7: Mean (a) haemoglobin, (b) reticulocyte percentage and (c) platelet counts in mice treated with four BU doses (see key) at fortnightly intervals. $n=5$ (d 1, 49, 85, 384); $n=10$ (d 140, 177, 210, 253, 485/497); *significantly different to control value, $p<0.05$; ** $p<0.01$; * $p<0.001$. In (b), significant increases in reticulocyte percentage were seen in the 40 mg BU/kg group at d 49 and 140, and in the 25 mg/kg group at d 253. Significant decreases were seen at d 384 in the 25 mg/kg group ($p<0.05$) and in the 40 mg/kg group ($p<0.01$).**

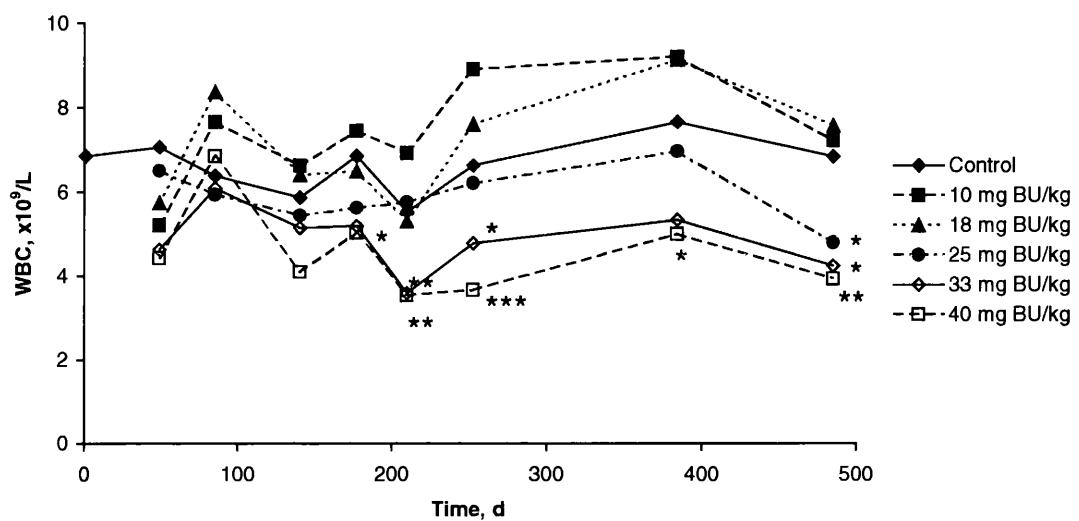
5.3.2.3 Leucocytes

Immediately after the final BU dose on d 49, the total leucocyte count was depressed in all BU treatment groups, counts showing a dose-related trend. (Fig. 5.8 (a)). However, on d 85, counts in all BU-treated groups in general were higher than on d 49. On d 85 at 25, 33 and 40 mg BU/kg, the WBC was close to the control value whilst at 10 and 18 mg/kg it was 20 % and 31 % higher than the control value, respectively. At d 140, counts in all BU-treated groups had fallen again and were all close to the control value, with the exception of the 40 mg BU/kg group which was only 69 % of the control value. From d 140 to d 485 the WBC of the 40 mg/kg group remained significantly depressed, at between 57 % and 74 % of the control value. Leucocyte counts in the 33 mg/kg group were also significantly depressed on most occasions from d 177 onwards, but to a slightly lesser extent than in the group receiving 40 mg/kg. The WBC of the 25 mg/kg group was slightly below the control mean on all occasions, except d 210, but only at d 485 was this reduction significant. At d 140, 177 and 210, leucocyte counts in the 18 mg/kg group did not differ greatly from the control mean, but from d 253 onwards, there were slight elevations in this dose group. At 10 mg BU/kg, the count remained elevated from d 85 onwards, although at no time was this elevation significantly different from the control value.

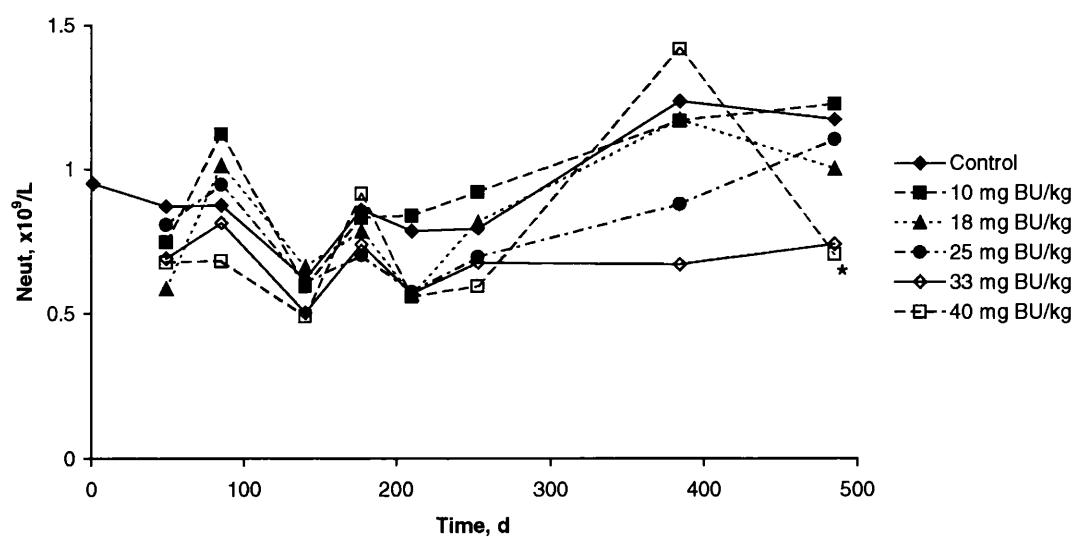
Neutrophil counts showed the same pattern of changes as the total WBC at d 49, 85 and 177 but thereafter the counts were depressed slightly on most occasions at the highest dose levels in comparison with the controls (Fig. 5.8 (b)). Neutrophil myeloperoxidase content (MPXI) showed no consistent pattern of alteration. Monocyte and eosinophil counts were also depressed at the highest BU levels throughout the study (but not at d 85

or d 485) and dose-responsiveness was more clearly demonstrated in these cell types than in the neutrophil count.

(a)



(b)



(c)

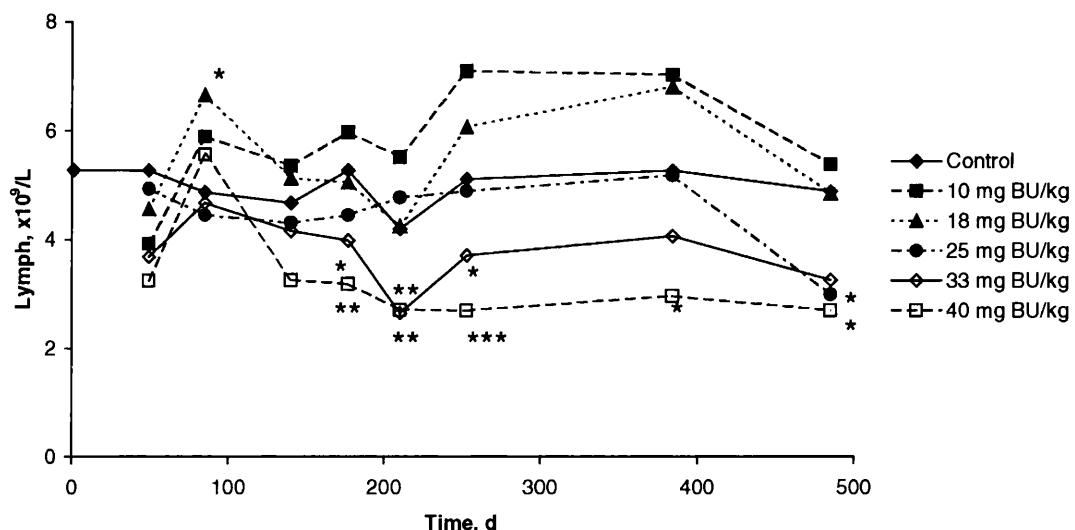


Figure 5.8: Mean (a) total leucocyte, (b) neutrophil and (c) lymphocyte counts in mice treated with four BU doses (see key) at fortnightly intervals. n=5 (d 1, 49, 85, 384); n=10 (d 140, 177, 210, 253, 485/497); *significantly different to control value, p<0.05; **p<0.01; ***p<0.001.

The pattern of change in lymphocyte counts (Fig. 5.8 (c)) was very similar to that seen in the total leucocyte count. Lymphocyte counts in the 40 mg/kg group were reduced to about 50 percent of control values on all occasions with the exception of d 85 and an intermediate effect was seen at 33 mg/kg. Elevations of the lymphocyte count were seen at 10 mg/kg and to a lesser degree at 18 mg/kg. In addition to lymphocyte count reductions, the LUC count was depressed from d 140 onwards in the 40 and 33 mg BU/kg groups. However, no elevation of LUC counts was present at lower BU dose levels, as had been seen in the lymphocyte counts.

A preliminary examination of lymphocyte subtypes on d 384 revealed a selective effect on B (IgG positive) cells alone. At 33 and 45 mg BU/kg, statistically significant reductions of IgG positive cells to 24.6 % and 15.7 % of the control mean, respectively, were observed. T cell CD4⁺ and CD8⁺ subsets were unaffected. Lymphocyte subset analysis on d 485 (Fig. 5.9) confirmed the findings of d 384. On this occasion, the mean IgG positive cell counts in the 25, 33 and 40 mg BU/kg dose groups were 45.8 %, 47.0 % and 39.5 % of the control mean count, respectively. These reductions were statistically significant ($p<0.05$), whilst the mean total T cell (Thy 1.2⁺) and CD4⁺ and CD8⁺ cell counts in BU-treated mice did not differ significantly from the control mean values.

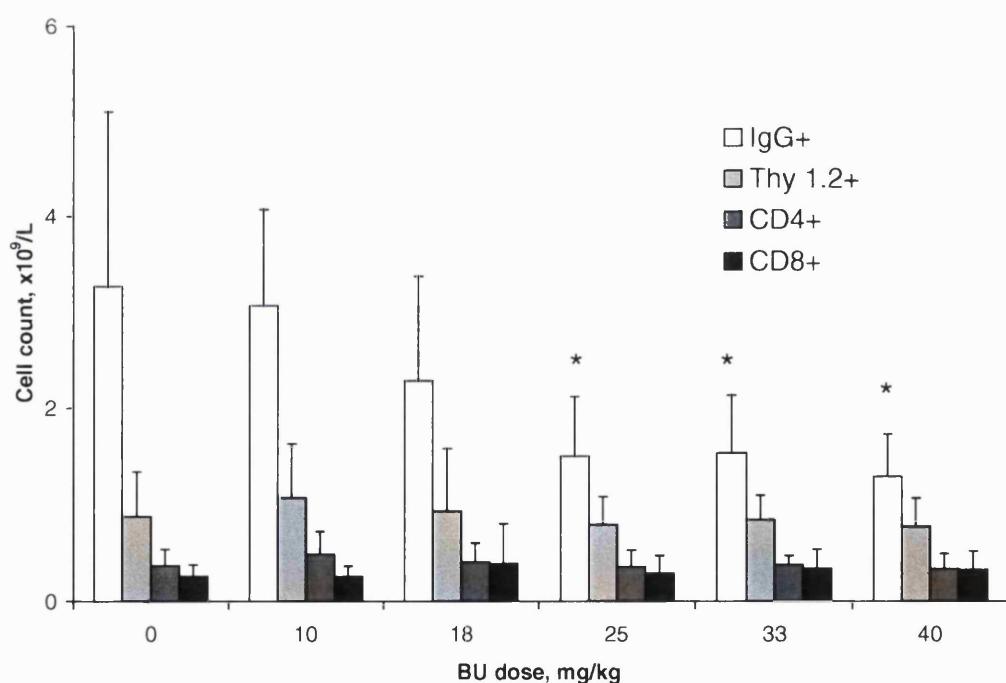


Figure 5.9: Mean lymphocyte subset counts on d 485 in mice given four BU doses.
 n=5; *significantly different to control value, $p<0.05$.

5.3.3 Histopathological Findings

5.3.3.1 Three Animals with Marrow Aplasia, Sampled on Days 107, 129 and 168

The sternal marrow of three mice (no. 20, 22 and 23, Table 5.2 (b)) sampled on d 107, 129 and 168 showed complete loss of haemopoiesis and large cystic spaces were observed within the marrow compartment (Fig. 5.1 (f)), although the femoral marrow count in one animal (no. 23) was normal. The spleens of these three animals showed only slight to moderate erythropoietic activity and abundant haemosiderin (Fig. 5.10 (b)) was present in the spleen of two animals (no. 20, 23). Splenic myelopoiesis and megakaryopoiesis was unremarkable in all three animals. Lymphoid depletion was present in the mesenteric lymph nodes of one mouse (no. 20; Fig. 5.10 (e)); sinus histiocytosis and marked haemosiderosis was also evident. The cervical lymph nodes of one animal (no. 22) also showed sinus histiocytosis. Slight bronchial associated lymphoid tissue (BALT) activity was evident in the lungs of one animal (no. 22). No abnormalities were present in the eyes of these three animals.

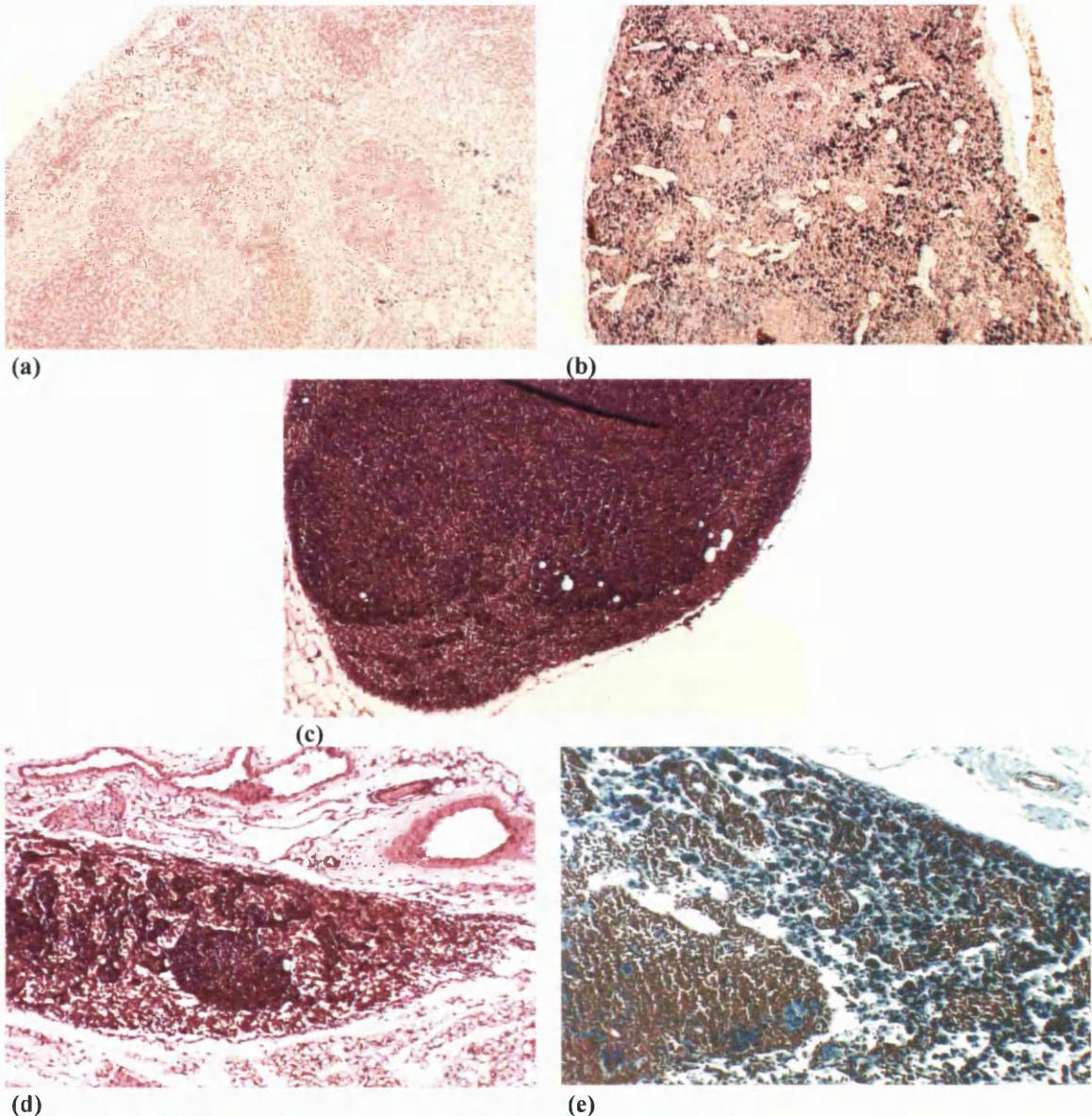


Figure 5.10: Lymphoreticular abnormalities in aplastic ICD mice in Experiment 6.

- (a) Control, d 85: spleen, Perl's stain and eosin $\times 90$. Positivity for haemosiderin (blue) is graded as very slight.
- (b) ICD, d 168 (Table 5.2 (b), no. 23): spleen, Perl's stain and eosin $\times 90$. Abundant haemosiderin is present throughout the red pulp.
- (c) Control, d 85: mesenteric lymph node, H&E $\times 90$. Germinal centres are visible in the cortical area.
- (d) ICD, d 107 (Table 5.2 (b), no. 20): mesenteric lymph node, H&E $\times 90$. Marked lymphoid depletion with sinus histiocytosis.
- (e) ICD, d 107: mesenteric lymph node, Perl's stain and Martius Scarlet Green $\times 180$. Evidence of erythrophagocytosis is present in macrophages which are strongly positive for iron.

5.3.3.2 Four Animals with Lymphoma, Sampled on Days 175, 184, 191 and 217

Normal lymph node (cervical and mesenteric) architecture was lost in four animals sampled on d 175, 184, 191 and 217 (Table 5.2 (c), no. 25 to 28), with diffuse infiltration of lymphoma cells throughout all compartments (cortex, paracortex and medullary sinuses; (Fig. 5.11 (a)). Normal thymus structure was also lost in all four animals with involvement of both the cortex and medulla (Fig. 5.11 (f)). There was also a loss of normal splenic architecture in these animals with diffuse infiltration of lymphoma cells throughout both the white and red pulp (Fig. 5.3 (e)). In the lungs of all four animals there was infiltration and replacement of BALT with lymphoma cells (Fig. 5.11 (b)) and there were also perivascular and subpleural accumulations of neoplastic cells. The livers of all four animals were severely affected with large deposits of lymphoma cells in many of the portal regions and there were also deposits adjacent to some central veins and some smaller accumulations within the sinusoids (Fig. 5.11 (c)). In all animals except mouse no. 25 the sternal marrow cavity (Fig. 5.3 (c)) was full of lymphoma cells with few or no recognisable haemopoietic cells. However, in mouse no. 25, lymphoma cell infiltration was present in the femoral marrow. There was often involvement of adjacent intercostal muscles. Lymphoma cells were seen infiltrating tissues adjacent to the eye (Fig. 5.11 (d)) especially around the optic nerve. Focal haemopoiesis was present in the cervical lymph nodes (Fig. 5.11 (e)) of one animal (no. 25), and the spleens of all four animals showed moderate or marked erythropoietic activity. The cervical lymph nodes of one animal (no. 28) showed sinus histiocytosis. Glycogen vacuolation was slight or marked in the livers of three mice, but in general the livers, apart from the presence of tumour cells, were unremarkable. Similarly, apart from tumour cell deposition, the lungs of these four mice were otherwise normal and no abnormalities were present in the eyes. The lymphoma cell appeared similar in all four animals (Fig. 5.3).

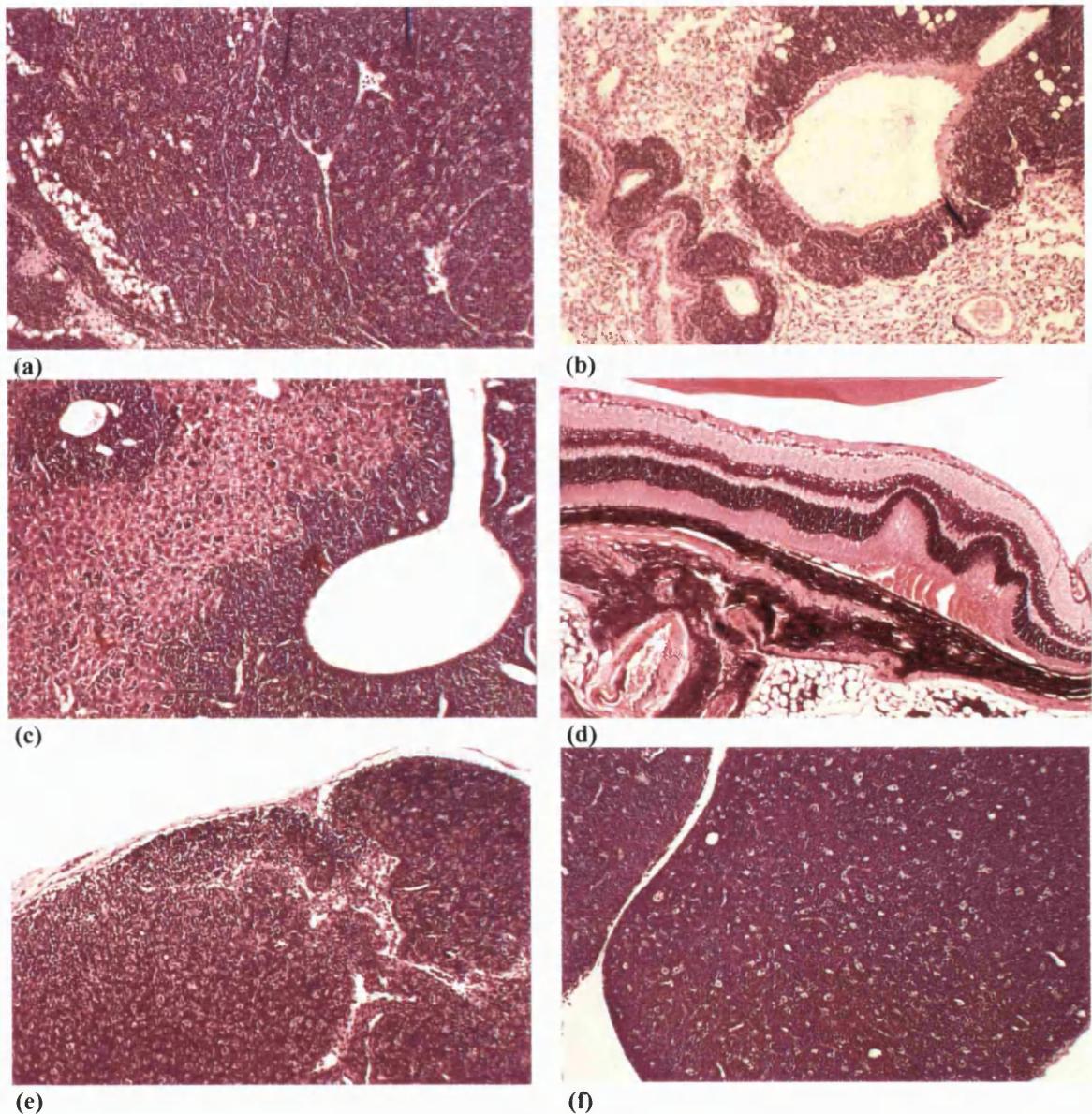


Figure 5.11: Tissue infiltration in lymphoma bearing ICD mice in Experiment 6.

- (a) ICD, d 184 (Table 5.2 (c), no. 26): mesenteric lymph node, H&E $\times 90$. Diffuse lymphoma cell infiltration with loss of normal architecture.
- (b) ICD, d 184: lung, H&E $\times 360$. Lymphoma cell infiltration of BAL and perivascular region.
- (c) ICD, d 191 (Table 5.2 (c), no. 27): liver, H&E $\times 360$. Diffuse lymphoma cell deposits are present adjacent to the portal tract (left) and central vein.
- (d) ICD, d 184: eye, H&E $\times 90$. Lymphoma cell infiltration of periocular areas.
- (e) ICD, d 175 (Table 5.2 (c), no. 25): cervical lymph node, H&E $\times 90$. In addition to lymphoma cell infiltration, focal haemopoietic activity is evident in the subcapsular region.
- (f) ICD, d 191: thymus, H&E $\times 90$. Diffuse lymphoma cell infiltration is accompanied by a "starry sky" appearance due to the presence of scattered histiocytes.

5.3.3.3 Scheduled Autopsy at Day 85

Tissues were examined at this timepoint from five animals in each of groups 1 to 4 (these animals had been dosed at 0, 10, 18 and 25 mg BU/kg (Table 5.1)). The only notable changes in the tissues of these mice were minor signs of lymphoid hypoplasia in the lymph nodes of animals treated at 25 mg BU/kg, but these effects were within the range of background variability for this strain of mouse.

5.3.3.4 Scheduled Autopsy at Day 384

At d 384, tissues were examined from five mice each in groups 1 to 6. These animals had been dosed at 0, 10, 18, 25, 33 and 40 mg BU/kg and had received CAPS in the drinking water from d 85 onwards (Table 5.1). Lymphoma involvement was noted in the thymus, spleen and mesenteric lymph nodes of two animals receiving 40 mg BU/kg and one receiving 25 mg/kg. A hepatocellular adenoma (Fig. 5.12 (a)) was present in one animal receiving 33 mg BU/kg. Sternal bone marrow cellularity appeared slightly reduced in one animal receiving 40 mg BU/kg, and megakaryocyte activity in the sternal marrow was decreased in the majority of BU-treated animals at all dose levels. There was slight or marked myeloid hyperplasia (Fig. 5.12 (b)) in the sternal marrow of three animals receiving 40 mg BU/kg. Splenic myelopoiesis was marked (Fig. 5.12 (c)) in one 40 mg BU/kg animal, but this response was very slight or slight in the other BU-treated groups and in the controls. The incidence of moderate or marked erythropoiesis (Fig. 5.12 (d)) appeared slightly increased in the spleens of BU-treated animals in all groups, and three 40 mg/kg mice and one 25 mg/kg animal had slight congestion of the red pulp.

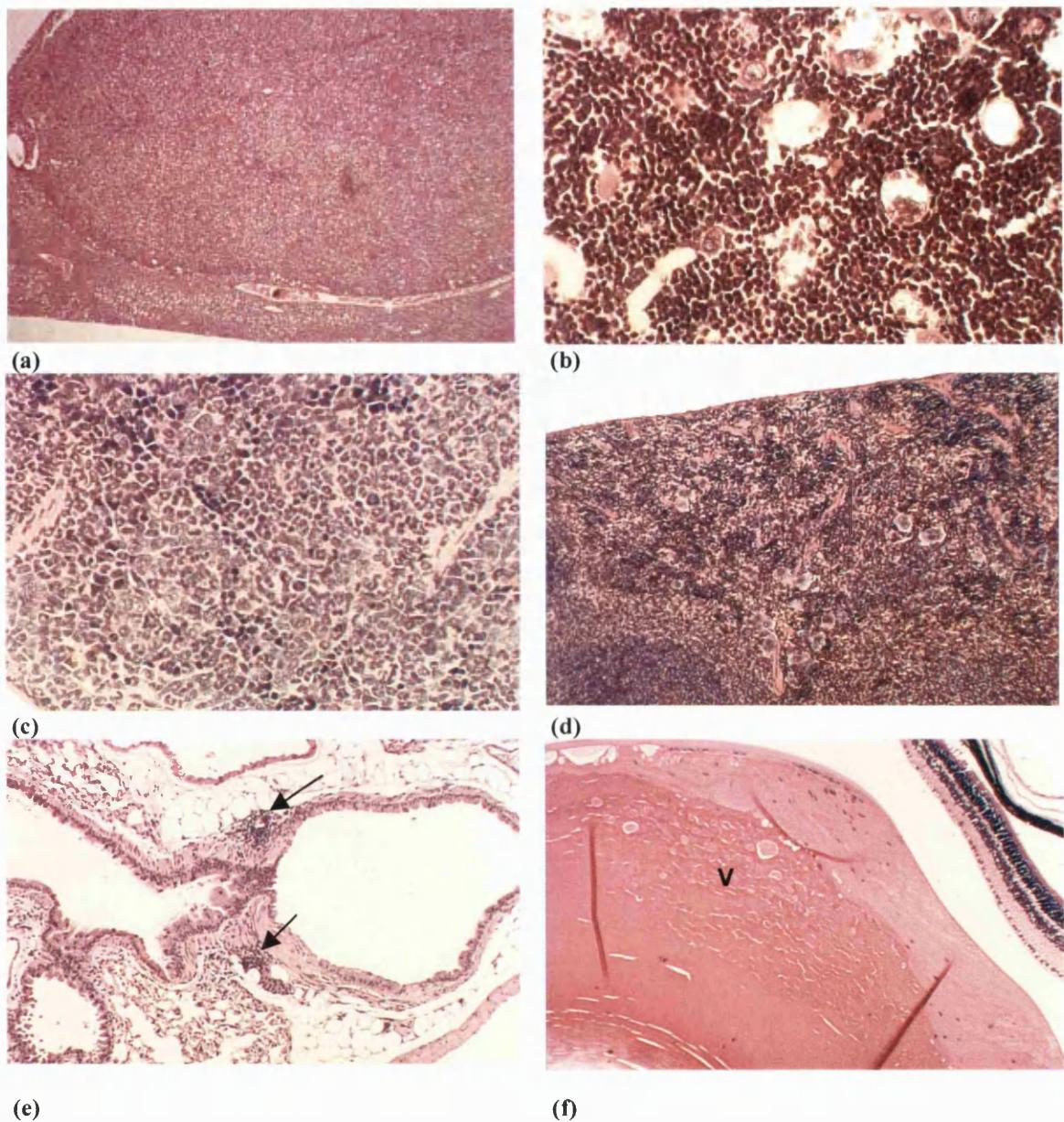


Figure 5.12: Histological abnormalities in BU-treated mice at the scheduled autopsy on d 384 of Experiment 6.

- (a) 33 mg BU/kg: liver, H&E $\times 90$. A large hepatocellular adenoma is seen in the upper portion of the section.
- (b) 40 mg BU/kg: sternal marrow, H&E $\times 360$. Marked myeloid hyperplasia.
- (c) 40 mg BU/kg: spleen, H&E $\times 360$. Marked erythroid hyperplasia.
- (d) 40 mg BU/kg: spleen, H&E $\times 90$. Marked erythroid hyperplasia with megakaryocytosis is present.
- (e) 40 mg BU/kg: lung, H&E $\times 90$. Slight BALT activity (arrowed) persists in this section. In other animals autopsied in this dose group, BALT was not detected.
- (f) 40 mg BU/kg: eye, H&E $\times 360$. Marked lenticular vesiculation (V) is present.

Lymphoid depletion and lymphoid hyperplasia in the mesenteric lymph nodes was noted in some animals receiving 33 and 40 mg BU/kg, but not in the controls or those mice receiving 10, 18 or 25 mg BU/kg. Histiocytic hyperplasia was seen in the mesenteric lymph nodes of one animal in the 40 mg/kg group. In the cervical lymph nodes, sinus histiocytosis was seen in one to four animals per group at 10, 18, 33 and 40 mg BU/kg; this change was not seen in the controls. Lymphoid hyperplasia in the cervical lymph nodes was evident in one control mouse, in one animal in the 10 mg BU/kg group, and in two animals in the 18 mg BU/kg group, but not in animals receiving higher doses of BU. The thymus of two animals in the 40 mg/kg group showed lymphoid hyperplasia, as did one control mouse, and one in the 18 mg BU/kg group. Lymphoid depletion in the thymus was present in one animal receiving 25 mg BU/kg, but not in the controls or in other BU-treated animals. In the control and BU-treated animals, the livers showed a similar distribution of glycogen vacuolation and portal infiltrates of mononuclear cells, with the exception of one animal treated with 18 mg BU/kg, where localised glycogen vacuolation was present. In the liver, the incidence of very slight parenchymal mononuclear cell foci was similar (1 to 3 animals per group) in the controls and in the 10 and 18 mg BU/kg groups, but this change was absent in animals receiving 25 to 40 mg/kg of BU. Liver parenchymal necrosis was present in one animal per group in those receiving 25 to 40 mg BU/kg, but necrosis was not seen in the control mice or in those receiving 10 or 18 mg/kg. In the lungs, the distribution of BALT (Fig. 5.12 (e)) appeared reduced in animals receiving 40 mg BU/kg. In three animals treated with 40 mg/kg, BALT was not seen, whereas activity was very slight or slight in the controls and in all other BU-dosed animals. Also in the lungs, moderate or marked perivascular mononuclear cell infiltrates were present in three animals at 18 mg BU/kg, whilst this finding was slight or very slight in the controls and in all other BU-dosed animals. The

eyes of one animal receiving 33 mg BU/kg, and two receiving 40 mg BU/kg, showed lenticular vesiculation (Fig. 5.12 (f)), and in one of the latter group the lesion was bilateral. No abnormalities were detected in the eyes of the control animals nor in any other of the BU-treated animals.

5.3.4 Chloramphenicol and Chloramphenicol Succinate Analysis

The estimated consumption of CAPS in mice based on mean body weight and water consumption per mouse at four timepoints are shown in Table 5.8. Water consumption per mouse fell from 0.12 mL/g bodyweight/d immediately after CAPS administration began (on d 85) to 0.06 mL/g/d by d 482. The estimated CAPS intake therefore fell from approximately 400 mg/kg/d to approximately 200 mg/kg/d over the same period.

Levels of CAP in the serum were assayed on d 489 in a small number of animals from groups 1 to 7 (Table 5.9). CAP was found in only three of 15 serum samples taken from animals which had received CAPS in the drinking water at 4.0 mg/mL. The serum concentrations where CAP was present were 1.72, 0.34 and <0.55 µg/mL. In the other 12 samples, from animals which had received CAPS in the drinking water, no CAP was detected.

Stock solutions of CAPS and dosing solutions of CAPS in the water bottles were analysed for stability over a period of four consecutive days (0 to 72 h). This time period was equivalent to the time that the dosing solution in drinking water was left in the animal room before the water bottles were changed.

Table 5.8: Estimated consumption of CAPS in group 1 mice treated with BU vehicle followed by CAPS in the drinking water (4.0 mg/mL) from d 85.

Day	Mean body weight, g	Mean water consumption, mL/mouse/d	CAPS consumption, mg/kg/d
86	29.75	3.7	413.4
93	27.10	3.2	394.8
475	52.53	3.4	215.1
482	51.33	2.8	181.2

Table 5.9: Serum CAP levels in samples from individual mice killed on d 489. Results are means of duplicate analyses. *0.55 µg CAP/mL found in one duplicate sample only, the other sample being negative.

Group	BU dose, mg/kg	CAPS in drinking water, 4.0 mg/mL	Serum CAP level, µg/mL
1	0	Yes	0
	0	Yes	0
	0	Yes	1.72
2	10	Yes	0
	10	Yes	0
	10	Yes	0
3	18	Yes	0
	18	Yes	0
	18	Yes	0.34
4	25	Yes	0
	25	Yes	<0.55*
5	33	Yes	0
	33	Yes	0
	33	Yes	0
6	40	Yes	0
7	0	No	0
	0	No	0
	0	No	0
	0	No	0

Table 5.10: CAPS concentrations (mg/mL) in stock CAPS solution and dosing solution (drinking water). Results are means of duplicate analyses.

Time of analysis after preparation, h	CAPS concentration, mg/mL	
	100 mg/mL stock solution	4.0 mg/mL dosing solution
0	109.69	3.81
24	92.51	3.74
48	90.76	3.45
72	95.00	3.59

There were slight losses in the concentration of CAPS (Table 5.10) in both the stock solution and the dosing solution. The stock (100 mg/mL) solution concentration at 0 h was 109.69 mg/mL and at 72 h was 95.00 mg/mL. The lowest concentration measured was 90.76 mg/mL at 48 h after preparation. All these concentrations were therefore within 10 % of the formulated concentration. The dosing solution concentration was 3.81 mg/mL at 0 h and 3.59 mg/mL at 72 h, with the lowest concentration, measured at 48 h, being 3.45 mg/mL. Whilst this last figure is within 10 % of the 0 h concentration, 3.45 mg/mL is 13.9 % below the formulated concentration of 4.0 mg/mL.

5.4 Discussion

The purpose of BU treatment in the present study (Experiment 6) was to induce injury to haemopoietic stem cells as proposed by Morley and others (Morley and Blake, 1974a; Morley *et al.*, 1975; 1978a; Morley, 1980). This injury, it was considered, might confer susceptibility to CAPS (Morley *et al.*, 1976b) and induce late marrow aplasia, and mirror the idiosyncratic reaction to CAP seen in man (Yunis and Bloomberg, 1964; Yunis, 1973; Chaplin, 1986b). However, no haemotoxic effects could be shown in Experiment 6 with CAPS administered in the drinking water at 4.0 mg/mL.

Analysis of lymphoma ICDs showed no excess hazard with respect to CAPS (Table 5.3; S. Pattenden, personal communication), which contrasts with the finding of Robin *et al.* (1981), who found that lymphoma deaths were three times as frequent in mice given BU and CAPS, compared with those which received BU alone.

Analysis of group mean haematology data at scheduled timepoints showed no significant differences between animals receiving BU and CAPS and those which received BU alone (Table 5.4, 5.5; Fig. 5.6).

CAP could only be detected in the serum of three of 15 animals sampled at d 489 (Table 5.9). The possibility that CAPS was breaking down in the water bottles during the period of administration was considered but was excluded because only slight reductions in concentrations occurred over a 72 h dosing period (Table 5.10).

Nevertheless, BU was effective in inducing persistent peripheral cytopenias (Fig. 5.6, 5.7,

5.8, 5.9) and reduced splenic cellularity in surviving animals (Table 5.7; Andrews *et al.*, 1997; 1998). Survival in BU-treated mice was related to BU dose: at the maximum dose administered (40 mg/kg), significant toxicity was demonstrated as survival was about 41 % at 497 d (Fig. 5.4). This figure compares with approximately 50 % survival at 300 d in male CBA mice given two ip doses of 25 mg BU/kg (Alexander and Connell, 1960) and 50 % survival at 520 d in female RF mice given four fortnightly ip doses of BU at 12 mg/kg (Conklin *et al.*, 1965). BU-related mortality in the present experiment was from two causes. In the first 168 d of the experiment, animals dying were markedly cytopenic with hypoplastic bone marrow (Table 5.2a and b), and from d 174 until the end of the experiment, nearly all animals dying had developed a T cell lymphoma (Table 5.2 (c) and (d)).

Histopathological evidence of damage to lymphoid organs was seen at the scheduled autopsy at d 84 and 385 (Section 5.3.3) with both lymphoid depletion and lymphoid hyperplasia being present in BU-treated mice. Evidence of persistent damage to stem cells of other organ systems was seen as the appearance of fur greying, previously reported as a result of BU administration (Botnick *et al.*, 1979; Down *et al.*, 1989), and also lenticular vesiculation in the eye (Dunn, 1974).

Studies have demonstrated the effect of interactions of CAP with other haemotoxic agents. Haem and protein synthesis are inhibited in rabbit reticulocytes *in vitro* in an additive fashion when CAP is incubated with isoniazid or ethanol (Gruenspecht *et al.*, 1979). Four weeks' concurrent treatment of rabbits with BU and CAP significantly enhanced the granulocytopenia induced by BU alone (Lu *et al.*, 1956/7). However, Speck and Moeschlin (1968) reported no additional haemotoxic effect on rabbits given 10 mg

BU/kg/d and 1 g CAPS/kg/d for two weeks. Irradiated mice and rats showed decreased rates of erythropoiesis, ^{59}Fe incorporation and granulopoiesis when CAP is subsequently administered, but these effects were reversible (Stojanovic and Zelenika, 1967; Pospíšil *et al.*, 1980; Vácha *et al.*, 1981).

Attempts to reproduce the findings of Morley *et al.*, (1976b) have also been reported. Pazdernik and Corbett (1980) and Robin *et al.*, (1981) administered CAPS or CAP to mice using a preparatory BU regime identical to Morley *et al.*, (1976b). Two weeks after the last BU injection, CAPS was given in the drinking water at 5 mg/mL by Pazdernik and Corbett (1980), and the effects on haemopoiesis were measured after six weeks. In the work by Robin *et al.* (1981), a total of 1200 mg CAP/kg was injected ip over five weeks, 20 weeks after the cessation of BU treatment, and haematological assessment was made 18 weeks later. As in the present study, in neither report (Pazdernik and Corbett, 1980 ; Robin *et al.*, 1981) was AA found as a consequence of treatment with both drugs.

However, Morley *et al.*'s hypothesis, that residual BU-induced stem cell damage could be used to demonstrate haemotoxicity by other drugs (Morley and Blake, 1974a; Morley *et al.*, 1976b) was validated by Boyd *et al.* (1982). In this study, captopril, which is associated with neutropenia and agranulocytosis in a minority of patients, but well tolerated in rats and mice at levels of up to 100 times the human dose, was administered in the diet at 450 mg/kg/d for 26 weeks to mice, previously given ip BU at 20, 20, 20 and 10 mg/kg at two-weekly intervals. These mice showed a greater than 50 % reduction in CFU-GM with BU and captopril, compared with BU alone, although CFU-S and peripheral counts were unaffected. Deaths from marrow aplasia or lymphoma were not reported (Boyd *et al.*, 1982).

The BU-induced reductions in peripheral cell counts, notably of leucocytes, (Fig. 5.8 (a)), B lymphocytes (Fig. 5.9) and platelets (Fig. 5.7 (c)) in the present study compares with those reported by Morley and Blake (1974a) who reported slight, but significant reductions in haematocrit and leucocytes, but no reduction in platelet count 61 to 175 d after the cessation of BU administration. Similarly, Boyd *et al.* (1982) reported a slight reduction in haematocrit, and a 50 % reduction in neutrophils and CFU-GM at 26 weeks after BU treatment. A reduction in B lymphocytes was also noted by Morley *et al.* (1974b). However, the persistent elevation of the lymphocyte count after 10 mg BU/kg (Fig. 5.8 (c)), which was not associated with an increase in LUC count (Andrews *et al.*, 1997) does not appear to have been reported elsewhere. Robin *et al.* (1981), on the other hand, found no effect on peripheral counts in BU-treated mice at 300 d after treatment, which is in agreement with data reported by Morley *et al.* (1978a).

The reduction in splenic cellularity to 71.2 % with 40 mg BU/kg at d 85 (Table 5.7) compares with a reduction to 59 % at six weeks after BU treatment by Pazdernik and Corbett (1980). Pazdernik and Corbett also reported a reduction in femoral marrow cellularity to 83 % at this time; in the present study a slight but non-significant reduction in femoral cell count was present at 40 mg BU/kg at d 49, but not subsequently (Table 5.6). Similarly, Wathen *et al.* (1982) reported a reduction in femoral cell count to 60 % six months after four weekly ip injections of 20 mg BU/kg, with CFU-S and CFU-GM reduced to 10 % and 25 % respectively. Fried and Adler (1985) also report a reduction in CFU-S to 21 % at 52 weeks after four weekly ip injections of BU at 12 mg/kg, which compared with reductions to 10 - 40 % in CFU-S and CFU-GM levels reported by Morley *et al.* (Morley and Blake, 1974b; Morley *et al.*, 1975; 1978a).

The foregoing illustrates that, in the present study, BU caused persistent damage to the haemopoietic system, but also that the haematological response to BU shows inconsistencies between reports in the literature. However, in neither the current study, nor in the reports of Pazdernik and Corbett (1980) or of Robin *et al.* (1981) was it demonstrated that CAPS (or CAP) was capable of causing AA in mice with a pre-existing BU-induced haemotoxic lesion. Indeed, Robin *et al.* (1981) reported the development of lymphomas in CAF₁ mice treated with BU, BU and CAP, and CAP alone, whilst no control mice had developed lymphomas by d 350 of their study (20 mg BU/kg was administered ip on d 1, 15, 29 and 43 and 100 mg CAP/kg ip 5 d/week from d 183 to 218). BU-only and CAP-only treated mice developed lymphomas at a similar rate (4 of 35 BU-treated mice and 2 of 41 CAP-treated mice), but BU + CAP treatment induced lymphomas in 13 of 37 animals. Similarly, Sanguineti *et al.* (1983) reported lymphomas in BALB/c mice and C57BL/6N mice when CAP was given in the drinking water at 2000 ppm for two years. Lymphomas were found in 12 % of CAP-treated BALB/c mice (controls, 3 %) and in 23 % of C57BL/6N mice (controls, 8 %). However, in the present study, using the B6C3F₁ mouse, CAP had no influence on the development of BU-induced, or spontaneously-occurring lymphomas (Table 5.3).

The development of BU-induced lymphoma from d 174 onwards (Table 5.2) probably reflects the spontaneous predisposition of the B6C3F₁ strain to this particular pathological outcome. Morley and Blake (1974a) reported the occurrence of lymphoma in about 3 % (15/494) of BU-treated Swiss mice. The incidence of spontaneous lymphoblastic lymphoma in female Swiss (CD1) mice is reported as 0 -5 % (Lang, 1995) and in the BALB/c strain, used by Morley in combination with CAPS treatment (Morley *et al.*,

1976b), 3 - 6.7 % (Frith *et al.*, 1983; Krueger, 1990). The B6C3F₁ strain, on the other hand, has a high lymphoblastic lymphoma incidence, of up to 45 % (Ward *et al.*, 1979; Wogan, 1980; Scales and Andrews, 1991). The localised pattern of cytoplasmic staining for α -naphthyl acetate esterase and acid phosphatase in lymphoma cells from ICD animals in Experiment 6 (Fig. 5.3 (b)) was indicative of T cell lineage (Krueger, 1990). The high susceptibility of the B6C3F₁ strain to 1,3-butadiene induced T cell lymphomas (57 %), relative to the lower incidence in NIH Swiss mice (14 %), has been ascribed to the presence of endogenous retroviruses in the B6C3F₁ strain (Irons *et al.*, 1989). An increased incidence of thymic lymphomas in the B6C3F₁ strain has also been reported following the administration of dideoxycytidine (ddC) (Sanders *et al.*, 1995) and phenolphthalein, and phenolphthalein is thought to promote leukaemogenesis by direct damage to the p53 tumour suppressor gene (Dunnick *et al.*, 1997).

Although cytochemistry indicated that the lymphoma in the present study was of T cell origin (Fig. 5.3 (b)), and infiltration was seen in the majority of tissues examined, it was not possible to state with certainty which organ might have been the site of origin for the neoplastic development. It has been suggested elsewhere that BU induces thymic lymphoma (Upton, 1961; Conklin *et al.*, 1965; Bhoopalam *et al.*, 1986). The median time of clinical appearance of lymphoma in the present experiment was 260 - 270 d after the commencement of BU treatment. Both the phenotype of the neoplastic cell and the rapid development of the lymphoma are in contrast to spontaneously occurring lymphomas in B6C3F₁ mice. In long-term background studies with this and other mouse strains, the median occurrence of lymphoma is greater than 500 d, and the cell type is predominantly of the B lineage (Frith *et al.*, 1985; Williams, personal communication). Depletion of lymphoid tissue in the thymus is a consistent feature in the development of thymic

lymphoma (Siegler, 1968) and has been reported in rodents as a consequence of cytotoxic treatment (Imai, 1990). In the present experiment, instances of lymphoid depletion were observed in lymphoid tissue including the thymus. Whilst lymphoid depletion at d 85 (in the 25 mg BU/kg group only) and at d 384, were within the background variability reported for the B6C3F₁ mouse, there was a sustained reduction in spleen weight and splenic nucleated cell count (Andrews *et al.*, 1998). Peripheral lymphocyte counts and LUC counts were consistently reduced at 33 and 40 mg BU/kg over the whole duration of the study (Fig. 5.8 (a), (c)), with a specific depletion of B cells (Fig. 5.9; Andrews *et al.*, 1997).

The development of aplasia followed by lymphoma (Table 5.2) is not inconsistent with the pattern seen in man, both in the natural history of "idiopathic" AA and in dyscrasias secondary to toxic chemicals or chemotherapeutic agents. The finding that MDS or AML occur in 16 % of patients at 10 years post immunosuppressive therapy (Young and Maciejewski, 1997) has lead some authors to postulate that AA is itself a preleukaemic condition (Marsh and Geary, 1991). In the evolution of MDS and AML secondary to chemotherapy a very high proportion of patients develop chromosomal abnormalities, most notably deletions or loss of chromosomes 5 and 7 (Wald and Conner, 1988; Levine and Bloomfield, 1992). The 5q31 site contains regions encoding for a number of cytokines responsible for the control of primitive haemopoietic stem cells including GM-CSF (Irons and Stillman, 1996). GM-CSF mediated proliferation of murine marrow cells *in vitro* is actually enhanced by BU pretreatment at very low concentrations. This might result in an increase in the size of the proliferating stem cell population *in vivo*, rendering it at greater risk from subsequent malignant transformation (Irons and Stillman, 1993).

It is unclear, however, whether the sequential appearance of aplasia in mice followed by lymphoma represents a natural evolution of one condition into the other. It may be that two separate processes are involved, with different latencies, and that these are developing in parallel. However, BU-induced aplasia occurs due to a progressive loss of haemopoietic stem cells and exhaustion of their proliferative potential (Morley *et al.*, 1975; 1978a) and impairment of the marrow's ability to repopulate the thymus is critical in the development of thymic lymphomas (Irons *et al.*, 1996; Humblet *et al.*, 1997). It is also possible that those mice that develop lymphoma do so having recovered from aplasia as a result of the growth advantage of an abnormal clone of stem cells which subsequently becomes malignant. A similar mechanism is proposed for the evolution of PNH from AA in man. PNH develops in up to 9 % of patients surviving AA, following successful immunosuppressive therapy (Young and Maciejewski, 1997).

The administration of high doses of BU to mice appears to induce four specific and distinct haematological conditions. Firstly, a transient suppression of all myeloid elements (Experiments 1 to 3); secondly, the late development of a hypoplastic condition analogous to AA in man; thirdly, a persistent suppression chiefly affecting leucocytes but in conjunction with macrocytic erythropoiesis, and fourthly, lymphoma. These observations appear to validate the model of aplasia and residual haemopoietic injury proposed by Morley *et al.* (Morley and Blake, 1974a; Morley *et al.*, 1975; 1978a; Morley, 1980) in the present studies in the B6C3F₁ mouse, but question the suitability of the use of this combination of mouse strain and this method of CAPS administration.

There are several possible reasons for the failure of CAPS to exert additional toxicity over the pre-existing BU-induced lesion. These include firstly, metabolic differences in the

strain of mouse used: the mice used by Morley *et al.* (1976b) were BALB/c, whereas Pazdernik and Corbett (1980) used BDF₁ (C56Bl/6 × DBA/2) mice, Robin *et al.* (1981) used CAF₁ (BALB/c × AF₁), and in the current study, B6C3F₁ (C57BL/6N × C3H/HeN) mice were used. It has been demonstrated that strains of mice differ widely in their response to CAP and other xenobiotics (Miller *et al.*, 1978; Festing, 1987). Secondly, the route of CAP or CAPS administration: it was possible, in the present study, that in spite of the apparently large amount of CAPS ingested, none was entering the bone marrow of the mice. This became a serious consideration when it was found that at d 489, CAP could only be detected in the serum of three of 15 mice sampled, so that the possibility arose that in Experiment 6, the mice had not been exposed to CAPS or CAP.

A fall in serum CAP concentration over time has been reported in children given iv CAPS (Nahata and Powell, 1983), and increased clearance of CAPS has been reported in children during concurrent administration of phenobarbitone, phenytoin and rifampin, probably as a result of induction of hepatic glucuronyl transferase (Black *et al.*, 1978; Krasinski *et al.*, 1982; Ambrose, 1984; Prober, 1985). Similarly, BU can alter the pharmacokinetics of the anticonvulsant phenytoin, resulting in lower efficacy (Hassan *et al.*, 1996). It was therefore considered possible that decreased CAP availability had taken place, although, since CAPS and BU were not administered concurrently, it would be difficult to postulate that this could have been due to an interaction with BU.

The likelihood of the breakdown of CAPS in the water bottles over the three-day dosing period was investigated; this suspicion was not substantiated (Table 5.10). Further investigations into the kinetics and haemotoxicity of CAPS in the B6C3F₁ mouse

therefore needed to be undertaken before a definitive evaluation of the mouse model of AA could take place. These investigations were carried out in Experiments 7, 8, 9 and 10.

CHAPTER 6: INVESTIGATIONS INTO CHLORAMPHENICOL KINETICS

6.1 Introduction

Administration of compounds in the drinking water in rodent toxicity studies is a convenient and effective method of drug delivery, and as rodents are nocturnal feeders, dosing in this fashion usually leads to peak serum concentrations in the early morning and troughs in the late afternoon (Yuan, 1995). On the basis of water consumption data from Experiment 6, it was calculated that animals receiving 4.0 mg CAPS/mL in the drinking water, in the two weeks immediately after the start of CAPS administration, were ingesting in excess of 400 mg CAPS/kg/d (Table 5.8). This figure fell to approximately 200 mg/kg/d during the period 475 to 482 d at the end of Experiment 6, as water intake fell with the increasing age of the animals. Therefore it was surprising to discover that no CAP could be measured in the serum of 12 out of 15 animals which were sampled at *post mortem* examination during the morning at the end of Experiment 6. The working practice in the Pathology Department at Glaxo Wellcome was for autopsies generally to be carried out in the morning between 0900 and 1200 h. The following experiments (Experiments 7, 8 and 9) were therefore designed to examine the fate of orally-administered CAPS, both when administered in the drinking water and when administered by gavage. In addition, these experiments would compare the bioavailability of oral CAPS dosing with iv delivery of the drug. Furthermore, in view of the lack of any differential or additive effect of CAPS administration on BU-induced haematological changes, it was important to determine how much, if any, of the administered CAPS dose was entering the bone marrow as succinate or whether it was metabolised to CAP and entering the marrow in this form.

6.2 Materials and Methods

Mice were obtained from the Small Animal Breeding Unit, Glaxo Wellcome Research and Development, Ware, Herts. CAPS dosing was as described in Chapter 2. Blood, bone marrow and spleen sampling and their analysis were as described in Chapter 2. Bone marrow and liver samples for the determination CAP and CAPS were prepared as described in Chapter 2. CAP and CAPS analysis was performed using Method 2, Chapter 2. Human and murine blood for determination of the haemolytic potential of dosing solutions was obtained as described in Chapter 2. Determination of haemolytic potential and osmolality were as described in Chapter 2.

6.2.1 **Experiment 7: Periodicity of Plasma Levels of Chloramphenicol and Chloramphenicol Succinate when Chloramphenicol Succinate was Administered in the Drinking Water.**

Three groups of 16 mice, four mice per cage, were given drinking water alone, or drinking water containing CAPS at 4.0 and 6.0 mg/mL *ad libitum*. The chosen dose levels were based on the findings of Experiments 4 and 5, and the finding in Experiment 6 that 4.0 mg/mL was well-tolerated and non-haemotoxic. After 14 d of CAPS administration, blood, bone marrow and liver samples were taken at 7pm, 12.20 am, 5.40 am and 11 am for the determination of CAP and CAPS. Full blood count, differential leucocyte count and reticulocyte count, femoral marrow and splenic cellularity were also determined at these timepoints. Water consumptions per cage were recorded daily, and were also calculated for the four time periods 11 am to 7 pm on d 14; 7 pm on d 14 to 12.20 am on d 15; 12.20 am to 5.40 am on d 15, and 5.40 am to 11 am on d 15. The determination of the amount of water consumed was achieved by measuring the consumption (water bottle weight) of a single cage per treatment group at each timepoint and subtracting the

consumption (water bottle weight) measured in the previous period(s). Body weights were measured on d 0, 3, 7, 10 and 15.

6.2.2 Experiment 8: Kinetics of a Single Intravenous Dose of Chloramphenicol Succinate

Six groups of 4 mice each were given iv 10, 25, 50, 100 and 250 mg CAPS/mL in distilled water (Water for irrigation, BP). Dosing solutions were administered at 10 mL/kg, resulting in dosages of 100, 250, 500, 1000 and 2500 mg CAPS/kg. Four further mice received iv saline alone at 10 mL/kg. All animals were killed 15 minutes after dosing, and blood, bone marrow and liver samples taken for the determination of CAP and CAPS as described in Chapter 6.2. Blood, marrow and spleen samples were taken for cell counts, full blood count, differential leucocyte count and reticulocyte count as described in Chapter 2. Osmolality and *in vitro* haemolytic potential of dosing solutions with murine and human blood were assessed with aqueous and saline formulations of CAPS at concentrations of 0, 10, 25, 50, 100, 250 and 500 mg CAPS/mL.

6.2.3 Experiment 9: Kinetics of Chloramphenicol Succinate Administered by Gavage

Three groups each of 20 mice were given CAPS by gavage at concentrations of 2.5, 5.0 and 10.0 mg CAPS/mL in Water for irrigation BP. The resulting administered doses were 25, 50 and 100 mg/kg, respectively, 100 mg/kg being the maximum daily recommended dose for oral or intravenous preparations of CAP in man (Parfitt, 1999). Dosing solutions were administered at 10 mL/kg. Two groups of 5 control animals were given Water for irrigation BP at 10 mL/kg. After 0.5, 1.0, 2.0 and 4.0 h, 5 mice from each CAPS dosed group were killed and samples of blood and bone marrow were taken for CAP and CAPS analysis. Five control mice were killed and sampled at 0.5 and 4.0 h only.

6.3 Results

6.3.1 Experiment 7: Periodicity of Serum Levels of Chloramphenicol and Chloramphenicol Succinate when Chloramphenicol Succinate was Administered in the Drinking Water

6.3.1.1 Clinical Observations and Body Weight

Animals given 0 (tap water only), and 4.0 and 6.0 mg CAPS/mL in tap water *ad libitum* for 14 d appeared to show no signs of toxicity. However, weight gain was significantly decreased on d 3 in the mice receiving 4.0 mg CAPS/mL (mean body weight 22.8 g, controls 23.5 g, $p < 0.05$), and at 6 mg/mL the decrease was more marked (mean body weight 22.1 g, $p < 0.01$), but after 7 d dosing and thereafter, the treated groups were not significantly different from the controls.

6.3.1.2 Water Consumption

In the 14 d period prior to blood sampling for serum CAP and CAPS levels in mice given 6.0 mg CAPS/mL, drinking water consumption was reduced to 54.5 % of control values ($p < 0.001$). Those receiving 4.0 mg/mL consumed 73.7 % of the control volume ($p < 0.01$). Over the period of blood and tissue sampling on d 14 - 15, water consumption was maximal in controls and animals receiving 4.0 mg CAPS/mL between 7pm and 12.20am, and slightly lower between 12.20am and 5.40 am (Fig. 6.1). In the period from 11am to 7pm, very little water was consumed by any group.

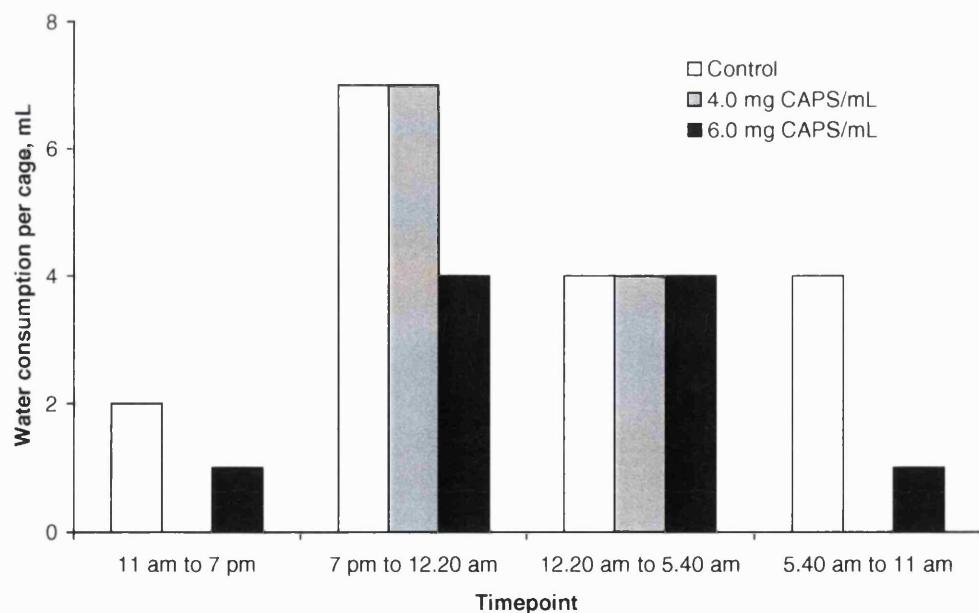


Figure 6.1: Water consumption, per cage of four mice, in mice receiving CAPS in the drinking water at concentrations as indicated. One cage per treatment group was measured at each timepoint and consumption in the previous measurement period subtracted. Where no data is shown, no water was consumed.

6.3.1.3 Serum Concentrations of Chloramphenicol Succinate and Chloramphenicol

6.3.1.3.1 Chloramphenicol Succinate

Levels of CAPS in serum showed a diurnal rhythm matching that of drinking water consumption (Fig. 6.2; Table 6.1). In the serum of mice receiving 6.0 mg CAPS/mL, CAPS was found in only two of the four mice sampled (4.2, 0.2 mg/L) at 7 pm; in three of the four samples at 12.20 am (4.3, 3.0, 6.7 mg/L) and in one of the four samples at 5.40 am (5.8 mg/L). At 4.0 mg CAPS/mL, CAPS was not detected in any sample at 7 pm, and in only one sample at 12.20 am and in one at 5.40 am (3.9, 4.4 mg/L, respectively). At 11 am, no CAPS was detected in any sample at either 4.0 or 6.0 mg/mL. No CAPS was detected at any timepoint in the serum of animals receiving tap water alone.

6.3.1.3.2 Chloramphenicol

CAP levels in serum (Fig. 6.3; Table 6.2) followed the same diurnal pattern as seen in the drinking water consumption (Fig. 6.1) and CAPS concentration (Fig. 6.2; Table 6.1). In both the 4.0 and 6.0 mg CAPS/mL groups, the maximum concentrations of CAP were detected at the 12.20 am timepoint. At this time, the mean CAP concentration at 4.0 mg CAPS/mL was 9.55 (range 0 - 27.7) mg/L and at 6.0 mg CAPS/mL, 3.20 (range 0 - 5.4) mg/L. CAP was present in only one of the four 11 am samples in each treatment group, 3.4 mg/L (4.0 mg CAPS/mL) and 2.7 mg/L (6.0 mg CAPS/mL). No CAP was detected at any timepoint in the serum of animals receiving tap water alone.

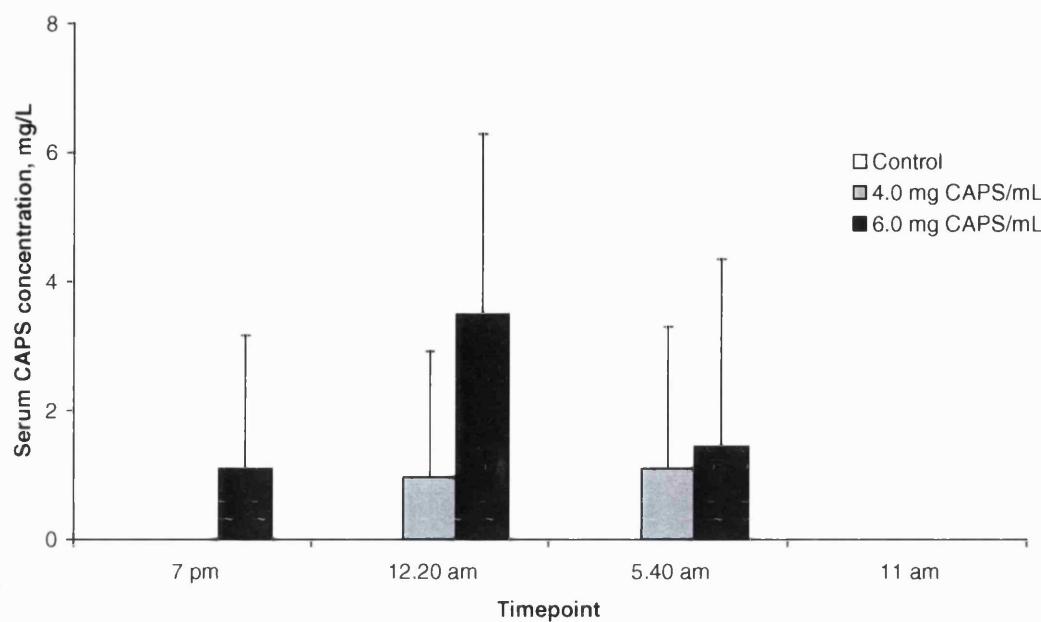


Figure 6.2: Mean and sd of serum CAPS concentrations in mice receiving CAPS in drinking water for 14 d at the concentrations indicated and sampled at four timepoints over a 16 h period.

Table 6.1: Serum CAPS concentrations (mg/L) in individual mice receiving CAPS in the drinking water for 14 d at the concentrations indicated and sampled at four timepoints over a 16 h period; 4 mice were sampled at each timepoint, in each treatment group.

Timepoint	CAPS dose, mg/mL		
	0	4.0	6.0
7 pm	0	0	0
	0	0	0
	0	0	4.2
	0	0	0.2
12.20 am	0	3.9	4.3
	0	0	3.0
	0	0	0
	0	0	6.7
5.40 am	0	0	0
	0	0	0
	0	0	5.8
	0	4.4	0
11 am	0	0	0
	0	0	0
	0	0	0
	0	0	0

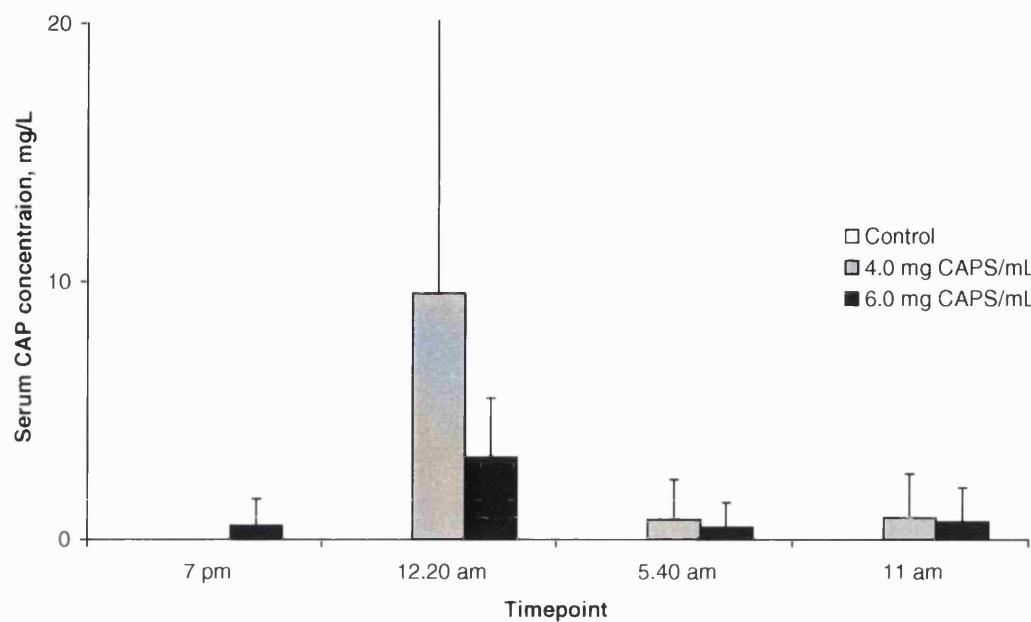


Figure 6.3: Mean and sd of serum CAP concentrations in mice receiving CAPS in the drinking water for 14 d at concentrations indicated and sampled at four timepoints over a 16 h period.

Table 6.2: Serum CAP concentrations (mg/L) in individual mice receiving CAPS in drinking water for 14 d at the concentrations indicated and sampled at four timepoints over a 16 h period; 4 mice were sampled at each timepoint, in each treatment group.

Timepoint	CAPS dose, mg/mL		
	0	4.0	6.0
7 pm	0	0	0
	0	0	0
	0	0	2.1
	0	0	0
12.20 am	0	5.9	5.4
	0	0	4.0
	0	27.7	0
	0	4.6	3.4
5.40 am	0	3.1	0
	0	0	0
	0	0	1.9
	0	0	0
11 am	0	0	2.7
	0	3.4	0
	0	0	0
	0	0	0

6.3.1.4 Haematology

Reticulocyte counts in control animals were similar at 7 pm, 12.20 am and 11 am, but showed a slight increase at 5.40 am (Fig. 6.4). Counts in mice receiving 4.0 and 6.0 mg CAPS/mL were not significantly different from the controls at 7 pm and 12.20 am, and both treatment groups also showed a slight increase in counts at 5.40 am when compared with the 7 pm values. The increases at 5.40 am were between 108.4 and 117.0 % of the 7 pm values but were not significantly different. At 11 am, the control and 6.0 mg CAPS/mL counts had returned to values close to the 7 pm counts. However, the 4.0 mg/mL count at 11 am was 120.9 % of control value, an increase which was significantly different from both the control value at 11 am and also the 4.0 mg/mL value at 7 pm ($p < 0.05$) for both analyses. At no other time point were reticulocyte counts in any treatment group different from the control at that timepoint or from the 7 pm values. No other diurnal or treatment-related changes were evident in other erythrocyte measurements.

There were apparent diurnal changes in the leucocyte counts and spleen cellularity in control animals (Fig. 6.5). The mean splenic nucleated cell count in controls at 12.20 am was not different from the 7 pm value, but at 5.40 am was 140.2 %, and at 11 am, 126.5 % of the 7 pm value, respectively. Both of these increases were significant (Fig. 6.5). The mean total leucocyte count, neutrophil and lymphocyte counts in control animals at 12.20 am were all similar to the 7pm values, but all showed highly significant increases at the 5.40 am and 11 am timepoints (Fig. 6.5). At 11 am, total leucocyte, neutrophil and lymphocyte counts were all greater than 300 % of the corresponding 7 pm value. However, at no timepoint were counts in any CAPS-treated group significantly different from the control value at the same time.

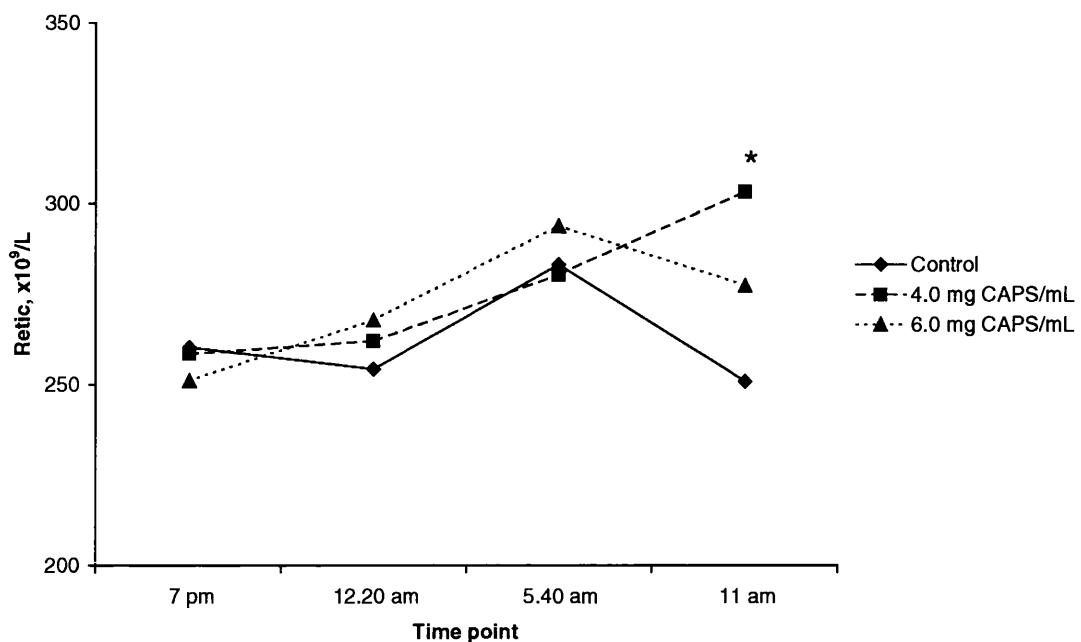


Figure 6.4: Mean reticulocyte counts in mice ($n = 4$) receiving CAPS in drinking water for 14 d and sampled at four timepoints over a 16 h period. * $p < 0.05$: significantly different from the control value at 11 am and from the 4.0 mg/mL value at 7 pm.

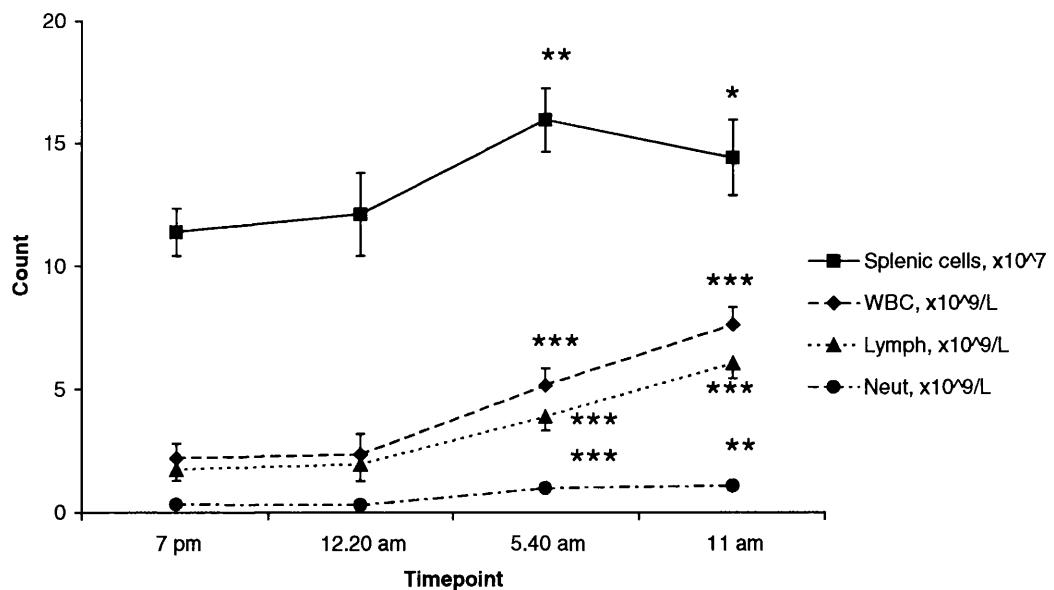


Figure 6.5: Mean and sd of spleen nucleated cell counts, total leucocyte, lymphocyte and neutrophil counts in control mice ($n = 4$) sampled at four timepoints over a 16 h period. * $p < 0.05$; ** $p < 0.01$; * $p < 0.001$: significantly different from 7 pm value.**

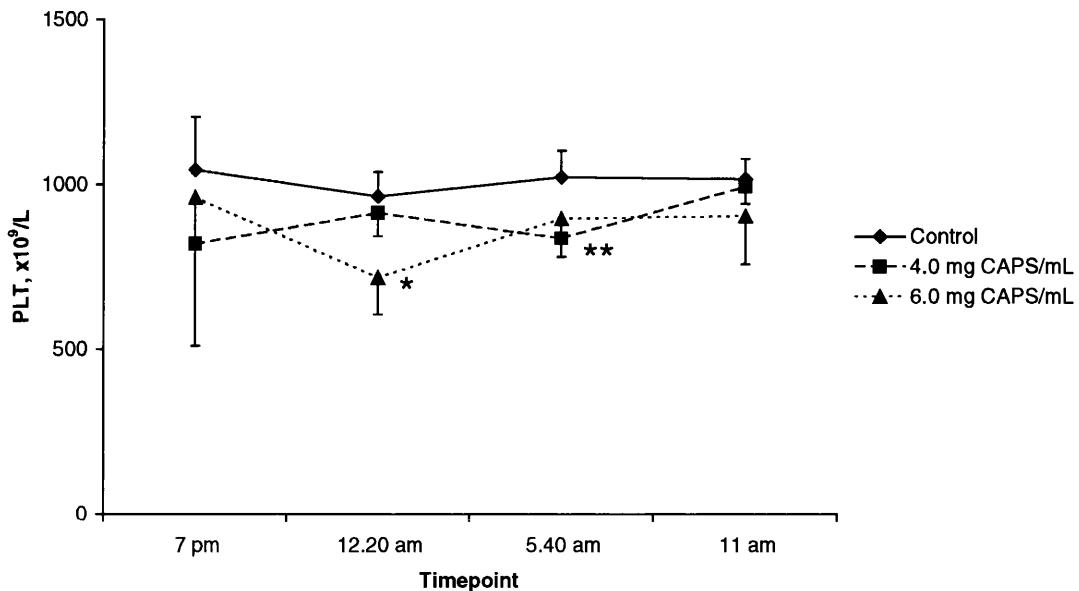


Figure 6.6: Mean and sd of platelet counts in mice (n = 4) receiving CAPS in drinking water for 14 d and sampled at four timepoints over a 16 h period. *significantly different to control value, p<0.05; **p<0.01.

Platelet counts in control mice showed no diurnal variation (Fig. 6.6), but in the 4.0 CAPS/mL group a significant depression was apparent at 5.40 am ($p<0.01$). A significant depression was also seen in the 6.0 mg/mL group at 12.20 am ($p<0.05$). However, considerable individual variability was present in the platelet counts in this study.

No morphological abnormalities were seen in the blood or marrow smears of animals treated with CAPS. Femoral counts showed no diurnal or dose-related effects.

6.3.2 Experiment 8: Kinetics of a Single Intravenous Dose of Chloramphenicol Succinate

6.3.2.1 Clinical Observations

Four mice per group were dosed iv with CAPS at 100, 250, 500 and 1000 mg/kg and these animals showed no ill effects. Mice given 2500 mg/kg suffered a transient loss of consciousness and were then subdued, with a low posture, unsteady gait and partially closed eyes. Two of four animals in this last dose group recovered and appeared well at 15 minutes after dosing. However, a third appeared unwell with continued laboured respiration and was killed. The respiration of the fourth animal was also laboured and it was found dead at 7 minutes post dosing. No samples were taken from the two last animals.

6.3.2.2 Haematology

Slight but significant reductions were seen in MCV at 1000 mg CAPS/kg, and in HDW at 500 and 1000 mg/kg (Table 6.3). Microscopy of stained smears showed crenation of erythrocytes in CAPS dosed animals at all dose levels (Fig. 6.7 (b)) and erythrocytes from animals given 2500 mg/kg showed slight spherocytosis. No significant effects were seen in any other erythrocyte parameter.

The reticulocyte percentage and absolute HFR count was significantly decreased at 100 and 1000 mg CAPS/kg, and the absolute reticulocyte count was significantly decreased at 1000 mg/kg (Table 6.3). However, the Sysmex R-1000 cytograms suggested a progressive decrease of erythrocyte volume as measured by forward light scatter (Fig. 6.7 (a)). This analytical effect appeared dose-related, and was so marked in samples from the two animals given 2500 mg CAPS/kg that the instrument was unable to

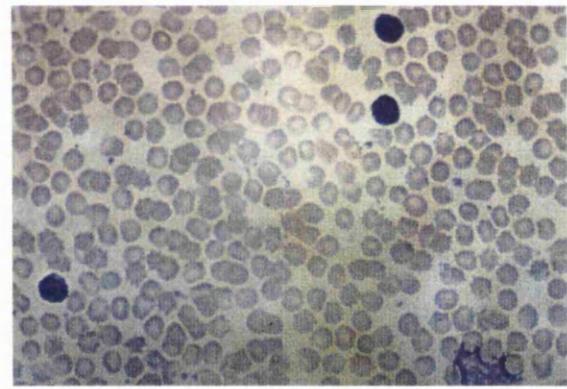
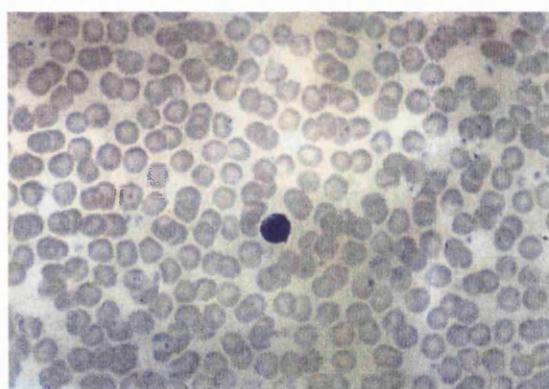
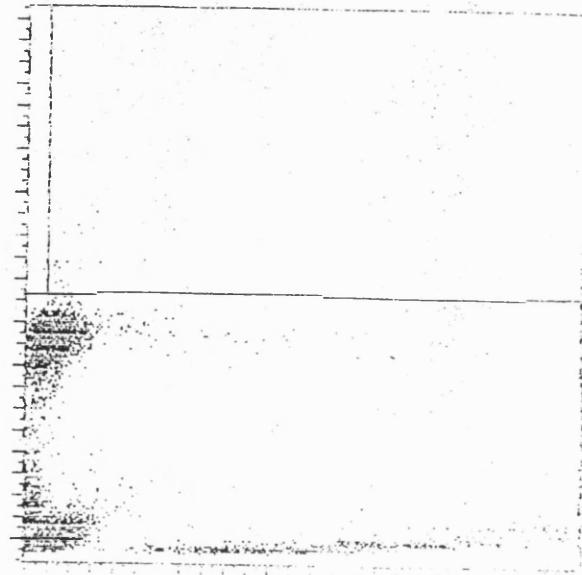
discriminate erythrocytes from noise, nor, therefore, reticulocytes from mature erythrocytes.

Table 6.3: Mean haematological measurements in mice 15 minutes after iv administration of CAPS, doses as indicated. n=4, except 2500 mg/kg, where n=2. Statistical analysis was not performed on data from this dose group. *significantly different to control value, p <0.05; ** p <0.01.

	CAPS dose, mg/kg					
	0	100	250	500	1000	2500
WBC, x10⁹/L	4.58	3.83	3.75	4.85	7.70*	12.43
RBC, x10¹²/L	9.13	9.23	9.45	9.17	9.34	9.20
Hb, g/dL	14.63	14.90	15.03	14.45	14.78	14.57
HCT,L/L	0.48	0.48	0.49	0.47	0.48	0.47
MCV, fL	52.10	51.73	51.40	51.10	50.93*	51.43
MCH, pg	16.00	16.20	15.85	15.73	15.80	15.80
MCHC, g/dL	30.73	31.33	30.90	30.88	31.03	30.80
PLT, x10⁹/L	901.75	762.25	889.00	891.50	709.25	478.67
RDW, %	11.55	11.63	11.55	11.33	11.45	11.57
HDW, g/dL	1.67	1.65	1.63	1.58**	1.60*	1.63
MPV, fL	4.50	4.35	4.50	4.55	4.75	5.03
NEUT, x10⁹/L	0.67	0.58	0.51	0.75	1.10	1.56
LYMPH, x10⁹/L	3.65	3.03	3.04	3.86	6.20**	10.28
MONO, x10⁹/L	0.10	0.07	0.06	0.07	0.15	0.13
EOS, x10⁹/L	0.12	0.13	0.09	0.10	0.17	0.26
BASO, x10⁹/L	0.02	0.01	0.01	0.04	0.02	0.04
LUC, x10⁹/L	0.04	0.02	0.03	0.02	0.04	0.09
MPXI	-20.00	-17.13*	-17.08*	-15.50**	-15.10*	-17.63
BM count, x10⁹/L	1.53	1.77	1.92	1.89	1.59	1.65
Spleen count, x10⁹/L	13.50	15.44	15.36	13.36	14.07	14.63
Retic %	2.25	2.01**	2.37	2.06	1.64**	-
Retic, x10⁹/L	195.93	177.10	212.35	178.75	126.98**	-
LFR, x10⁹/L	98.86	96.53	105.47	93.59	82.68	-
MFR, x10⁹/L	54.63	51.79	62.60	53.76	30.74**	-
HFR, x10⁹/L	42.43	28.79*	44.23	31.40	13.56***	-



(a)



(b)

Figure 6.7: Effects of iv dosing with CAPS. L: control; R: 2500 mg CAPS/kg.

- Sysmex R-1000 cytograms (reticulocyte analysis).** Vertical axis = forward light scatter (volume); horizontal axis = fluorescence intensity. At 2500 mg CAPS/kg there is an apparent reduction in erythrocyte volume, resulting in the placement of the lower discriminator such that erythrocytes and reticulocytes are classified as "noise".
- Erythrocyte morphology in May-Grünwald-Giemsa stained films.** Moderate crenation of erythrocytes is seen at 2500 mg CAPS/kg.

Platelet counts appeared to show a trend for reduction with increasing CAPS dose (Table 6.3), but there was considerable variability at some levels and no reductions achieved statistical significance. Increases were seen in leucocyte counts at 1000 and 2500 mg CAPS/kg, however, only the total leucocyte and lymphocyte counts at 1000 mg/kg were statistically significant. The neutrophil MPXI was significantly reduced in all CAPS-dosed groups. No changes were apparent in the cellularity of either the femurs or spleens. Bone marrow morphology in CAPS-dosed animals was unremarkable; no cytoplasmic or nuclear vacuolation was observed.

6.3.2.3 Osmolality and *In vitro* Haemolysis with Chloramphenicol Succinate Solutions

10 mg CAPS/mL in aqueous solution caused slight haemolysis with both human and murine blood (Fig. 6.8). However, haemolysis was not present at 10 mg CAPS/mL with blood from either species when CAPS was formulated in saline. Similarly, there was no haemolysis with blood from either species with CAPS in aqueous or saline solutions at 25, 50 or 100 mg/mL. However, marked haemolysis and colour change were present after the incubation of human and murine blood at 250 and 500 mg/mL.

Haemolysis of both human and mouse blood resulted after incubation with solutions whose osmolality was less than 100 mOsm/kg or greater than 685 mOsm/kg (Fig. 6.9), that is, aqueous CAPS solutions of 10 mg/mL and both aqueous and saline solutions of >100 mg CAPS/mL.

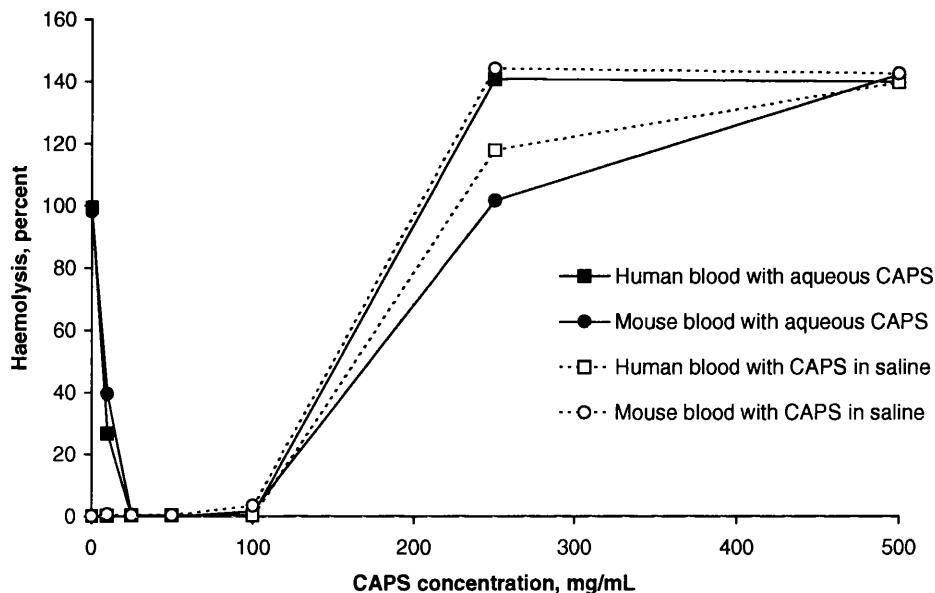


Figure 6.8: Mean percentage *in vitro* haemolysis of human and mouse blood with aqueous and saline CAPS solutions. Equal volumes of blood and CAPS solutions were incubated at 37°C for 45 min; haemolysis is expressed in relation to blood incubated with isotonic saline alone (0 % haemolysis) and 1 % aqueous saponin (100 % haemolysis). Atypical colour changes at high CAPS concentrations have caused measurements exceeding 100 %.

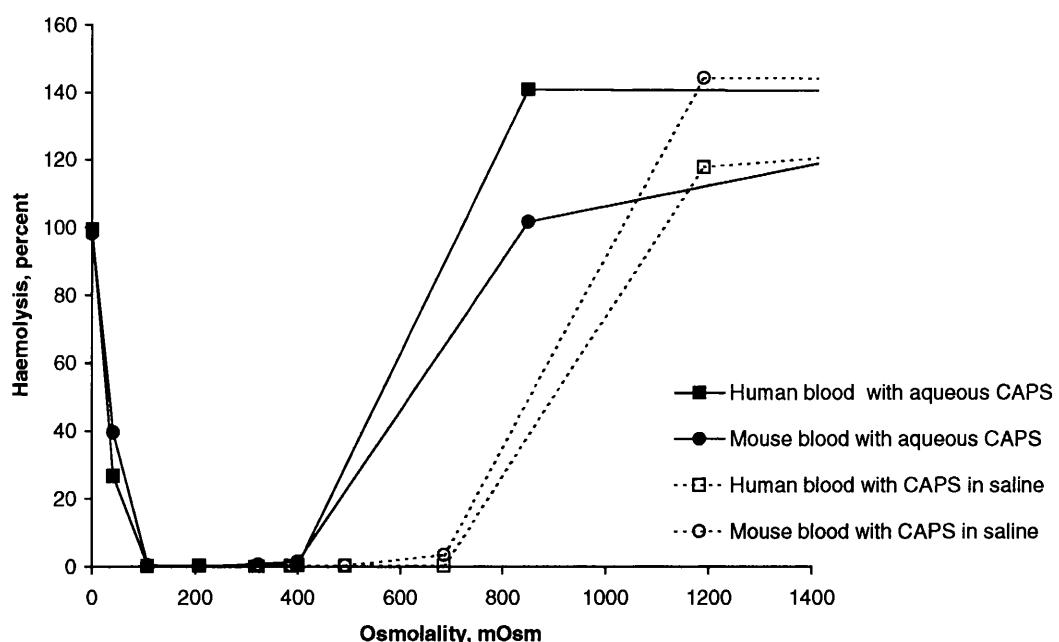


Figure 6.9: Mean *in vitro* haemolysis of human and mouse blood with aqueous and saline CAPS solutions plotted against the osmolality of the CAPS solutions. The horizontal axis (osmolality) is truncated for clarity.

The osmolality of the aqueous dosing solutions of CAPS ranged from 41 mOsm/kg (10 mg CAPS/mL) to 2175 (500 mg CAPS/mL; Fig. 6.10), and of saline CAPS solutions from 323 mOsm/kg (10 mg CAPS/mL) to 3086 mOsm/kg (500 mg CAPS/mL). The osmolality of distilled water and of isotonic saline was 0 mOsm/kg and 318 mOsm/kg respectively.

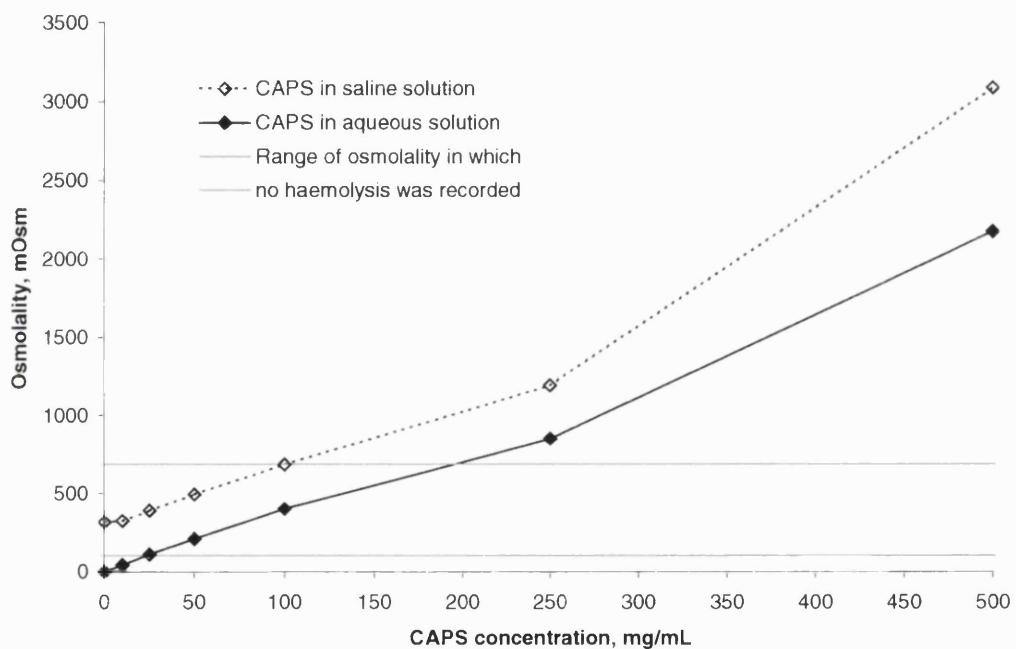


Figure 6.10: Osmolality of saline and aqueous solutions of CAPS. The range of osmolality in which no haemolysis was recorded, with either human or murine blood (100 to 685 mOsm/kg), is shown as parallel horizontal lines.

6.3.2.4 Serum, Liver and Bone Marrow Chloramphenicol Succinate and Chloramphenicol Concentrations

Concentrations of CAPS in the serum at 15 min after iv administration of 100, 250, 500, 1000 and 2500 mg CAPS/kg reflected the concentration of CAPS administered (Fig. 6.11; Table 6.4). Levels rose to a mean of 1326.7 mg/L in mice given 2500 mg CAPS/kg. Mean liver concentrations remained low and were similar at all dose levels, and not dose-related, with values of 6.90 µg/g (250 mg CAPS/kg), 11.65 µg/g (500 mg/kg) and 10.72 µg/g (2500 mg CAPS/kg). CAPS was only found in one of the four marrow samples in the 1000 mg CAPS/kg group (7.9 mg/L) and in one of the two samples in the 2500 mg CAPS/kg group (8.3 mg/L). CAPS was not detected in marrow samples from any other dose group.

Mean CAP levels in serum and liver were dose-related (Fig. 6.12; Table 6.5) and approximately equivalent at each dose level. In the serum, mean concentrations of 22.63, 64.58, 117.75, 255.73 and 357.80 mg CAP/L were found in samples from mice dosed at 100, 250, 500, 1000 and 2500 mg CAPS/kg, respectively. In liver samples, the mean concentrations for these CAPS dose groups were 21.07, 52.32, 138.50, 292.34 and 345.37 µg CAP/g, respectively. A dose-related trend was also apparent in bone marrow, with mean concentrations of 0.53, 0, 1.82, 5.21, and 15.06 mg CAP/L, respectively.

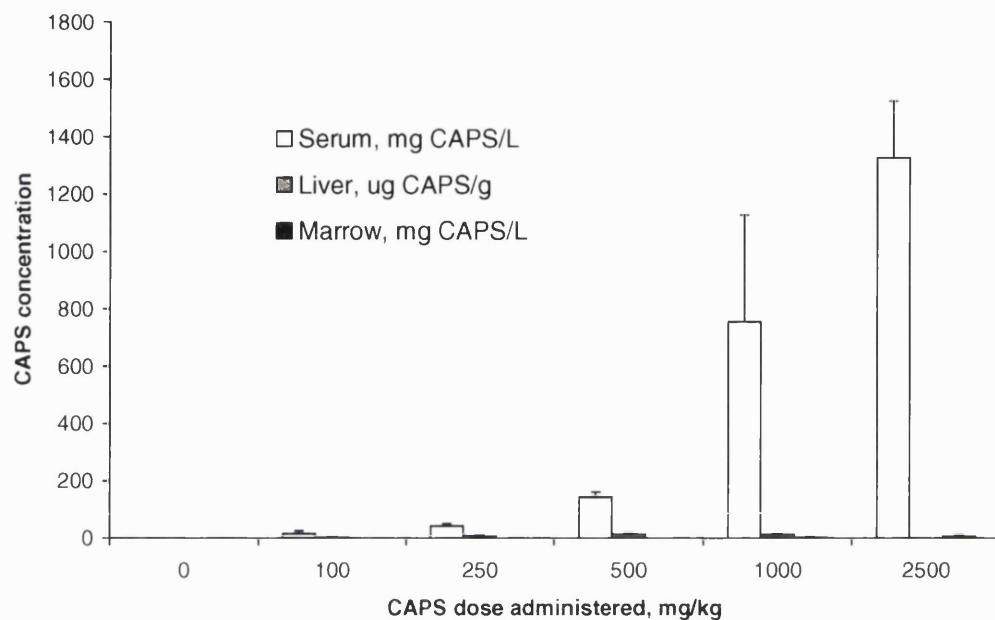


Figure 6.11: Mean and sd of concentration of CAPS in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS, dose levels as indicated. n=4, except 2500 mg/kg, n = 2. One serum sample at 100 mg/kg was lost, as was one liver sample at 2500 mg/kg.

Table 6.4: Individual concentrations of CAPS in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS, dose levels as indicated.

CAPS dose administered, mg/kg						
	0	100	250	500	1000	2500
Serum, mg/L	0		35.0	145.2	876.2	1467.6
	0	7.4	46.5	160.6	1151.6	1185.8
	0	25.0	34.7	143.9	262.0	
	0	10.0	48.4	116.3	731.6	
Liver, µg/g	0	0	9.52	10.08	14.54	0
	0	0	6.02	12.28	8.64	
	0	0	2.29	18.05	15.48	
	0	5.19	9.76	6.17	4.21	
Marrow, mg/L	0	0	0	0	7.93	0
	0	0	0	0	0	8.29
	0	0	0	0	0	
	0	0	0	0	0	

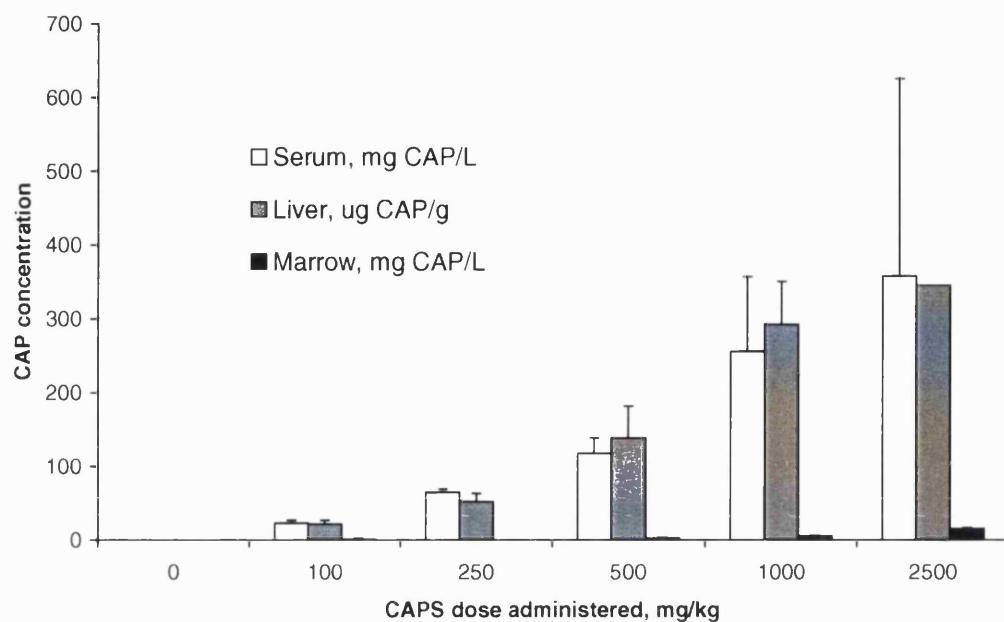


Figure 6.12: Mean and sd of concentration of CAP in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS, dose levels as indicated. n=4, except 2500 mg/kg, where n=2. One serum sample at 100 mg/kg was lost, as were one liver sample each at 1000 and 2500 mg/kg.

Table 6.5: Individual concentrations of CAP in serum, liver and femoral bone marrow of mice 15 min after a single iv dose of CAPS, dose levels as indicated.

		CAPS dose administered, mg/kg					
		0	100	250	500	1000	2500
Serum, mg/L	0	0	61.4	94.1	303.3	546.8	
	0	19.4	65.5	135.1	331.4	168.8	
	0	26.2	60.9	135.9	107.8		
	0	22.3	70.5	105.9	280.4		
Liver, µg/g	0	15.67	50.48	142.86	352.73	345.37	
	0	28.97	65.66	119.02	236.56		
	0	19.51	38.86	195.83	287.74		
	0	20.12	54.27	96.30			
Marrow, mg/L	0	0	0	2.05	6.19	15.58	
	0	0	0	2.05	6.19	14.54	
	0	0	0	3.19	3.38		
	0	2.13	0	0	5.08		

6.3.3 Experiment 9: Kinetics of Chloramphenicol Succinate Administered by Gavage

6.3.3.1 Clinical Observations

Animals appeared well at all times.

6.3.3.2 Serum and Bone Marrow Chloramphenicol and Chloramphenicol Succinate Concentrations

In mice given CAPS by gavage at 0, 25, 50 and 100 mg/kg and sampled at 0.5, 1.0, 2.0, and 4.0 h post dosing, serum CAP concentrations were maximal at 0.5 h (with the exception of the 50 mg CAPS/kg group), falling to undetectable levels at 4 h (Fig. 6.13; Table 6.6). In samples from mice dosed at 25 mg CAPS/kg, the mean concentrations at 0.5, 1.0, 2.0 and 4.0 h were 1.98, 0.48, 0.96 and 0 mg/L, respectively; in the 50 mg/kg group these values were 2.58, 2.70, 0.34 and 0, and in the 100 mg CAPS/kg group, 7.56, 3.94, 1.52 and 0 mg/L respectively. CAP was not detectable in controls at 0.5 and 4.0 h.

CAPS was not detectable in any serum sample from any CAPS-dosed group at any timepoint.

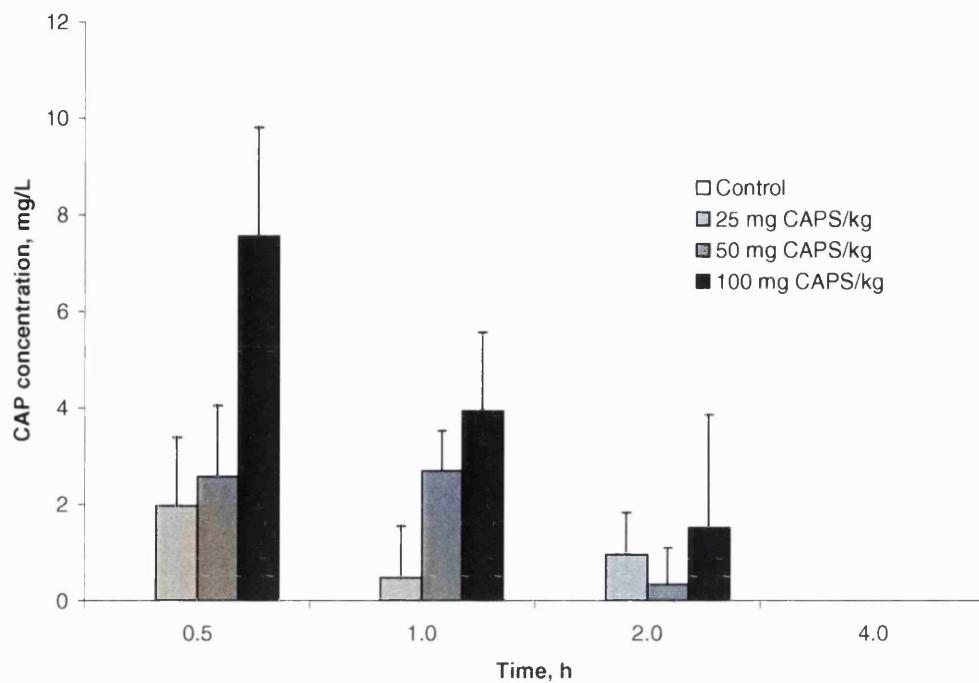


Figure 6.13: Mean and sd of serum CAP concentrations in mice following a single oral dose of CAPS, concentrations as indicated in figure. n=5.

Table 6.6: Individual serum CAP concentrations (mg/L) in mice following a single oral CAPS dose at the concentration indicated in the table. n=5; nd = not determined.

Time, h	CAPS dose, mg/kg			
	0	25	50	100
0.5	0	3.9	3.1	6.4
	0	1.5	0	5.6
	0	0	2.9	7.2
	0	2.3	3.3	7.2
	0	2.2	3.6	11.4
1.0	nd	2.4	1.5	3.7
	nd	0	3.7	4.5
	nd	0	3.0	1.5
	nd	0	2.3	4.0
	nd	0	3.0	6.0
2.0	nd	1.6	0	2.3
	nd	1.6	0	0
	nd	0	1.7	5.3
	nd	1.6	0	0
	nd	0	0	0
4.0	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0

CAP was detected in the bone marrow of all of the 5 mice receiving 50 mg CAPS/kg after 0.5 h, mean value 5.0, range 3.0 - 6.1 mg/L. Only two other marrow samples had measurable CAP levels: one mouse out of five at 25 mg/kg at 2 h post dosing had a level of 3.0 mg/L, and one out of five at 50 mg/kg at 1 h post dosing had a level of 2.6 mg/L. In all other marrow samples at 0.5, 1.0, 2.0 and 4.0 h, CAP was undetectable. CAP was not detected in the washings from any marrow sample, nor was there any obvious excess blood contamination in any group of samples.

CAPS was not detected in any marrow sample at any dose level at any time point.

6.4 Discussion

In Experiment 7, in which the variation in serum CAP and CAPS was investigated over a 24 h period, with the drug administered in the drinking water, a diurnal variation in drinking water intake was seen (Fig. 6.1). This result was expected for mice, which are nocturnal feeders. This pattern of water intake would be expected to lead to peak serum concentrations of the dosed compound being found between midnight and the early morning (Yacobi *et al.*, 1982; Yuan, 1995). However, this experiment demonstrated that although the peak serum concentrations of CAP (3-6 mg/L, in animals that drank water with 4.0 or 6.0 mg CAPS/mL) were present at midnight, by 11 am, only two of the sera from eight animals in the two dose groups contained measurable CAP (Table 6.2). This finding confirmed the observation in Experiment 5, where <2 mg/L CAP was found in the serum of only three of 15 animals sampled during the mid-morning on d 489 of the experiment (Table 5.9). Since comparable concentrations of CAP were found in the serum of mice drinking water with 4.0 mg CAPS/mL for 14 d (Experiment 6) and 489 d (Experiment 5), it would appear that increased clearance of the antibiotic with continued

administration does not take place in the mouse, unlike in children receiving iv CAPS (Nahata and Powell, 1983). Equally, the prior administration of BU in Experiment 5 did not appear to have affected the bioavailability of CAP.

Dosing of CAPS by the iv route at up to 2500 mg CAPS/kg body weight in Experiment 8 demonstrated that by 15 min after dosing, significant hydrolysis of CAPS to CAP had occurred (Fig. 6.11, 6.12). However, whilst CAP was found in the liver at concentrations comparable to that found in serum (Fig. 6.12), very little (approximately 1 - 2 % of the concentration identified in the serum or liver) was detected in bone marrow.

Oral dosing with CAPS (Experiment 9) demonstrated a time-related decay of CAP serum concentrations such that, after 1 h, animals receiving 50 mg CAPS/kg had serum CAP concentrations which were comparable to those reported by Morley *et al.* (that is, 2.5 and 4.0 mg CAP/L: Morley *et al.*, 1976b; Fig. 6.13; Table 6.6). However, by 2 h, CAP was detected in only one sample at this dose level and by 4 h, no CAP could be detected. In contrast to Experiment 7, where CAPS was administered in the drinking water at 4.0 and 6.0 mg/mL, serum from mice which were dosed by gavage at 2.5, 5.0 and 10.0 mg/mL in Experiment 9 contained no CAPS 30 min (nor at any other time) after dosing. This demonstrates rapid and complete conversion of CAPS to CAP when administered by the oral route. Nevertheless, similar levels of CAP were observed in the serum at 1 - 2 h after gavage dosing at 25 and 50 mg CAPS/kg (Table 6.6) and in mice drinking water containing 4.0 and 6.0 mg CAPS/mL (Table 6.2). From this it may be inferred that a constant, if low, serum concentration of CAP was maintained over the period of maximum drinking water intake in Experiment 6. The daily dosage of CAPS in Experiment 6 has been estimated at between 181.4 and 413.4 mg/kg (Table 5.8).

However, when 500 mg CAPS/kg was administered iv in Experiment 8, marrow was found to contain only 2.05 - 3.19 mg CAP/L at 15 min post dosing (Table 6.5). This low level of CAP in the marrow was achieved by iv dosing at 50 mg CAPS/mL, more than ten times the concentration of CAPS in the drinking water in Experiment 6.

A marked time-dependent fluctuation of leucocyte counts was noted in control mice sampled at four timepoints over 16 h (Fig. 6.5). This was in keeping with published observations in rodents (Brown and Dougherty, 1956; Berger, 1982) where lymphocyte numbers were inversely proportional to circadian cortisol levels (Ritchie *et al.*, 1983). It was also in step with the circadian periodicity of mitotic activity of murine bone marrow (Clark and Korst, 1969), which may have accounted for the slight increase in reticulocyte counts at 5.40 am (Fig. 6.4). However, the variation of splenic cellularity (Fig. 6.5) was unexpected. An age-dependent decrease in spleen weight which correlated with a concomittant drop in leucocyte count has been noted in mice (Fehér and Mózsa, 1974), however, this change occurred over a relatively long period, from 6 to 22 weeks of age.

Against the background of circadian haemopoietic activity in Experiment 7, there was little effect of CAPS dosing. The apparent increase in reticulocyte count in mice given 4.0 mg CAPS/mL in the drinking water at the 11 am sampling point (Fig. 6.4), and reductions in platelet count in CAPS-treated mice (Fig. 6.6) do not follow any specific pattern, and are considered to be probably spurious.

The slight reductions in MCV and HDW (Table 6.3), crenation and the appearance of microcytosis in the R-1000 cytograms (Fig. 6.7), after iv CAPS dosing in Experiment 8, are all consistent with the hypertonicity of the dosing solution which was confirmed by

increased *in vitro* haemolysis with increasing concentrations of the solutions (Mohondas *et al.*, 1989). The treatment-related reduction in reticulocyte count 15 min after dosing (Table 6.3) is difficult to explain as a physiological response, and this finding is more likely to be due to interference with the analytical process as a result of decreased binding of the fluorescent dye auramine O to RNA in the presence of significant circulating concentrations of CAP and CAPS. The elevations of neutrophil and lymphocyte counts with high dosage CAPS solutions (Table 6.3) was not unexpected, probably reflecting an acute adrenaline-mediated stress response, but the finding of slightly increased myeloperoxidase activity (MPXI) in neutrophils at all dose levels was also probably artefactual.

The lack of any CAPS-mediated haematological effects in the long-term study (Experiment 6) and in the present results (Experiments 7 to 9) threw into question whether the administration of CAPS via drinking water, with the resulting low serum concentrations, could be expected to exert any haemotoxicity in the B6C3F₁ mouse. Morley *et al.* (1974a) reported a 50 % inhibition of murine CFU-C *in vitro* at between 1-10 mg CAP/L. Miller *et al.* (1978) demonstrated inhibition of CFU-E at between 5 and 25 mg CAP/L in marrow cultured from various strains of mouse, but to cause a 50 % inhibition of myeloid colonies, it was found that concentrations of between 10 and 60 mg/L were necessary. Hara *et al.* (1978) reported similar concentrations for *in vitro* inhibition of murine erythroid and granulocytic progenitor cells, but were only able to demonstrate comparable reductions in *ex-vivo* femoral and splenic CFU-E and BFU-E after sc dosing for 5 d with 800 mg CAPS/kg/d. Firkin *et al.* (1974), on the other hand, who gave mice 600 mg/kg sc at 8 h intervals for 5 d, thus maintaining the serum CAP concentration at between 20 and 40 mg/L, reported increases in femoral and splenic CFU-

C. Whilst variations in culture technique may account for some of these differences, it is also recognised that different strains of mouse appear to vary in their sensitivity to CAP-induced haemotoxicity. In the work described by Miller *et al.* (1978), there was a more than 18-fold difference in inhibition of CFU-E between strains. Clearly, it may be necessary to administer relatively large doses of CAPS to mice of certain strains to cause significant haemotoxicity.

6.5 Parallel Collaborative Investigations with CAPS

As a direct result of the above findings (Experiments 4 to 9), a study was carried out where B6C3F₁ mice were administered CAPS at 0, 25, 50, 100 and 200 mg/kg by gavage. Marrow was removed for culture (CFU-E, BFU-E and CFU-GM) at 15 - 120 minutes after dosing (Holt *et al.*, 1998). *Ex-vivo* cultures from mice receiving a single dose of 100 mg CAPS/kg showed a 50 % inhibition of CFU-E growth where the marrow was sampled at 15 min post dosing. In mice receiving 50 or 200 mg CAPS/kg, 60 % inhibition of CFU-E growth was seen when marrow was sampled at up to 24 h after dosing. Erythroid precursors showed increased apoptosis 2 h and 24 h after treatment at 200 mg CAPS/kg. In later experiments, CD-1 mice were dosed daily by gavage at 200 to 2000 mg CAPS/kg/d for 7 d (Turton *et al.*, 1999). Animals remained well, though there was a trend for loss of body weight at the highest dose levels. In blood samples taken at 24 h after the final dose, at 2000 mg CAPS/kg, there was marked reticulocytopenia and reduction in femoral erythroid precursors, an increase in femoral myeloid numbers and slight thrombocytosis. BALB/c mice given a single dose of up to 6000 mg CAPS/kg by gavage showed only slight and temporary signs of toxicity (Robinson *et al.*, 1999; Turton *et al.*, 2000). These findings indicate that CAP can damage committed erythroid precursors at concentrations that are undetectable in the bone marrow, and that much higher doses of

CAPS than were used in Experiments 4, 5 and 6 would be needed to cause demonstrable reductions of peripheral elements. These findings also showed that high levels of CAPS can be dosed without significant toxicity.

CHAPTER 7: CHARACTERISATION OF THE MYELOTOXICITY INDUCED BY THE ORAL ADMINISTRATION OF HIGH DOSE LEVELS OF CHLORAMPHENICOL SUCCINATE

7.1 Introduction

The attempt to produce chronic bone marrow aplasia in the B6C3F₁ mouse was unsuccessful (Experiment 6). Whilst clear and persistent changes in peripheral blood and splenic cellularity could be attributed to BU dosing, the administration of CAPS in the drinking water was ineffective in superimposing aplasia on the BU-induced lesion. In Experiment 6, very low levels of CAP had been found in the serum of mice given CAPS in the drinking water and it was considered unlikely that sufficient drug was entering the marrow to induce a haemotoxic effect. Haematological changes seen in mice given CAPS alone without BU pretreatment (Experiments 4 and 5) could probably be attributed to inappetance and haemoconcentration. However, studies on the kinetics of CAPS in B6C3F₁ mice (Experiments 7 - 9) and experiments involving the high-dose administration of CAPS to other mouse strains (Turton *et al.*, 1999; 2000) showed that levels of CAP could be delivered, and haemotoxicity could be induced, when CAPS is administered by gavage. In general, the effects paralleled the haemotoxicity seen with CAP in man (Volini *et al.*, 1950; Scott *et al.*, 1965).

Yunis and Bloomberg (1964) observed that, in 37 of 46 cases of fatal marrow aplasia in man, the administration of CAP to the patient had been intermittent, not continuous, whereas in 36 cases of reversible marrow depression only in 8 had CAP been administered intermittently. It was therefore considered that an intermittent dosing regime in mice might be successful in inducing AA when administered against the background of BU-induced stem cell depletion.

Based on the findings in Experiments 7 - 9, and the results of parallel studies of CAPS haemotoxicity in five strains of mouse, CD-1, CBA, C3H, BALB/c and C57BL (Turton *et al.*, 1999, 2000; Festing *et al.*, 2000), a 25 d study was designed to examine the haemotoxicity of CAPS administered by gavage at 2500 and 3500 mg/kg/d for 5 d, followed by the observation of haematological parameters over the following 21 d. Evidence of haematological recovery by 30 d was expected, as Turton *et al.* had shown in mice given 1400 mg CAPS/kg/d for 10 d that there was a return to control values after 15 d (Turton *et al.*, 1999). Furthermore, in man, erythropoietic recovery occurs in reversible CAP toxicity at one week after the cessation of drug administration (Saidi *et al.*, 1961).

7.2 Materials and Methods

B6C3F₁ mice were obtained from Charles River, Margate, Kent. CAPS dosing was as described in Chapter 2 (gavage dosing). Blood, bone marrow and spleen sampling and analysis were as described in Chapter 2. Bone marrow progenitor cell assays were performed as described in Chapter 2.

7.2.1 Experiment 10: 25-day Gavage Study with High Dose Chloramphenicol Succinate

Three groups of twenty mice were dosed orally by gavage on d 0, 1, 2, 3 and 4 with CAPS at 0, 2500 and 3500 mg/kg. On d 5, 11, 18 and 25 (i.e., at 1, 7, 14 and 21 d post dosing), five animals per dose group were killed and samples of blood, femoral bone marrow and spleen were removed and full blood count, nucleated cell counts, femoral progenitor cell (BFU-E, CFU-E and CFU-GM) assays and microscopy of marrow smears and spleen touch preparations performed. Body weights were recorded twice weekly.

7.3 Results

7.3.1 Clinical Observations

Animals appeared well at all times. One animal in the group receiving 3500 mg CAPS/kg was killed on the first day of dosing following a dosing accident in which the gavage tube perforated the oesophagus (Fig. 7.1(a)).

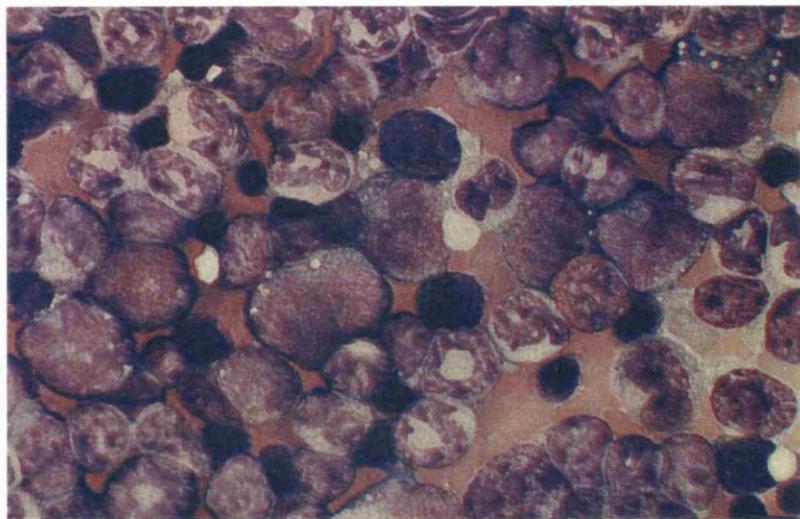
7.3.2 Haematology: Erythroid Series

Marked and highly significant reductions in erythocytes and their precursors were seen at 2500 and 3500 mg CAPS/kg on d 1 post dosing (Table 7.1).

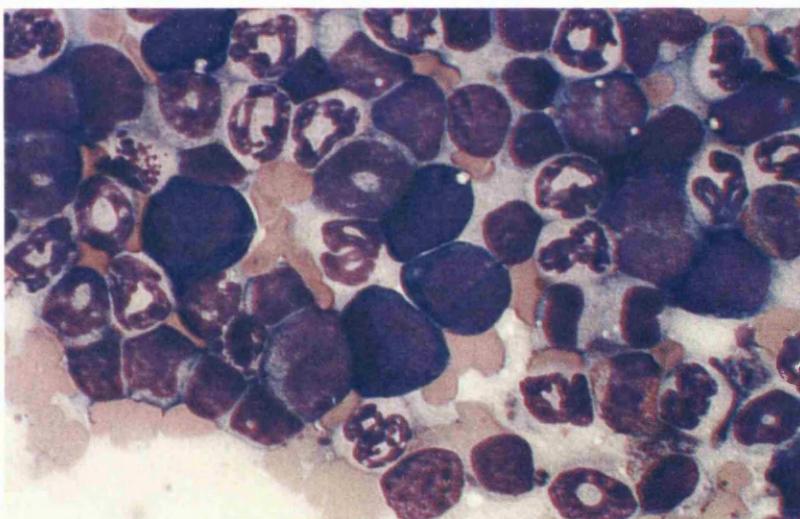
For example, on d 1, femoral erythroid cell count was reduced at 2500 and 3500 mg CAPS/kg to 20.8 % (p<0.001) and 9.5 % (p<0.001) of the control mean, respectively. In bone marrow smears at both dose levels, the only representatives of the erythroid series were pronormoblasts and early normoblasts, the majority of which contained a single large cytoplasmic vacuole (Fig. 7.1 (b)). On d 1, the splenic nucleated cell count was 57.7 % (p<0.001) and 43.0 % (p<0.001) of the control value at the 2500 mg/kg and 3500 mg/kg dose levels respectively, with an absence of erythroid activity as assessed from the examination of the touch preparations. Similar marked reductions of total and HFR reticulocytes were also seen at d 1. Red cell distribution width (RDW) and haemoglobin distribution width (HDW) were significantly reduced at this time, reflecting the depletion of immature circulating erythrocytes. Femoral BFU-E and CFU-E, erythrocyte count, haemoglobin and haematocrit were significantly reduced at both dose levels on d 1.

Table 7.1: Mean erythroid parameters following five consecutive daily CAPS gavage doses at 2500 and 3500 mg/kg. n=5, except at 3500 mg CAPS/kg on d 14 where n=4; for BFU-E, CFU-E, n=3; *significantly different to control value, p<0.05; **p<0.01; *p<0.001; nd = not determined.**

CAPS dose, mg/kg	Control				2500				3500			
	1	7	14	21	1	7	14	21	1	7	14	21
Hb, g/dL	15.45	15.38	15.13	15.36	13.93**	12.42**	15.24	15.58	14.16**	11.70**	15.53	14.86
RBC, $\times 10^{12}/L$	9.848	9.770	9.688	9.900	8.855**	8.058**	9.682	10.043	8.968**	7.642**	9.955	9.658
HCT, L/L	0.5423	0.5380	0.5325	0.5306	0.4800**	0.4476**	0.5354	0.5460	0.4872**	0.4110**	0.5450	0.5116
MCV, fL	55.05	55.06	55.00	53.60	54.23	55.58	55.30	54.35	54.32	53.78**	54.75	52.98
MCH, pg	15.70	15.74	15.60	15.52	15.70	15.42	15.74	15.50	15.78	15.32*	15.60	15.38
MCHC, g/dL	28.53	28.60	28.35	28.94	28.98	27.74	28.42	28.58	29.02	28.50	28.48	29.04
PLT, $\times 10^9/L$	847.3	970.4	1072.5	987.0	897.8	1261.8*	1254.0	1084.0	770.4	1765.0*	1066.0	973.0
RDW, %	11.60	11.84	11.85	11.66	11.10**	12.70**	12.86**	12.71**	10.92**	11.32	13.80**	13.26**
HDW, g/dL	1.500	1.514	1.558	1.492	1.430*	1.550**	1.622**	1.660**	1.392*	1.410**	1.665**	1.708**
Retic, $\times 10^9/L$	318.39	274.06	280.85	249.4	6.02***	414.1	275.38	249.2	6.08***	19.5***	397.75	212.56
HFR, $\times 10^9/L$	77.49	54.13	49.60	39.38	0.12***	170.83**	50.84	39.20	0.18***	1.48***	77.12	28.34
BM cells, $\times 10^7$	1.180	1.258	1.053	1.106	0.960	1.588	1.073	1.398	1.050	1.196	0.983	1.378
BM erythroid cells, $\times 10^7$	0.423	0.380	0.253	0.300	0.088***	0.558	0.345	0.400	0.040***	0.260	0.305	0.326
BFU-E per plate	42.67	nd	45.33	30.00	33.67**	nd	46.00	25.00*	26.33***	nd	46.33	28.33
CFU-E per plate	47.00	nd	48.67	60.00	35.00*	nd	49.67	60.00	33.33*	nd	39.00	58.67
Splenic cells, $\times 10^7$	8.68	8.82	9.82	9.91	5.01***	17.44***	11.94	11.86	3.73***	8.31	11.65	10.59



(a)



(b)

Figure 7.1: Vacuolation in bone marrow smears of mice receiving five daily doses of 3500 mg CAPS/kg by gavage.

- (a) ICD mouse on the first day of CAPS dosing. Vacuolation is especially noticeable in myeloid and monocytic precursors. MGG $\times 1200$.
- (b) Day 1 post dosing. Vacuolation is present in early normoblasts. MGG $\times 1200$.

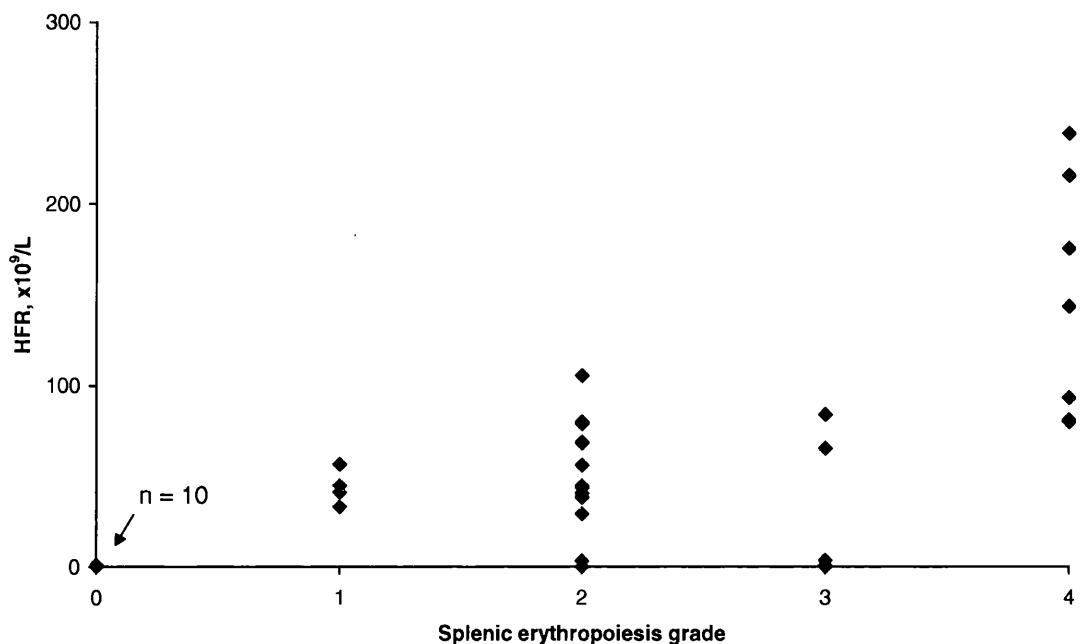


Figure 7.2: Comparison of splenic erythropoietic activity with HFR reticulocyte count in individual mice sampled at various timepoints after the administration of 3500 mg CAPS/kg. Splenic erythropoietic activity was graded in touch preparations: 0 = absent, 1 = very slight, 2 = slight, 3 = moderate, 4 = marked.

By d 7, at 2500 mg/kg, regeneration of erythrocyte production was evident from the marked erythropoietic activity in spleen touch preparations, and the significant increases in splenic nucleated cell count, HFR reticulocytes and RDW (Table 7.1; Fig 7.2). Total reticulocyte count and femoral erythroid count were elevated, but not statistically significantly at d 7.

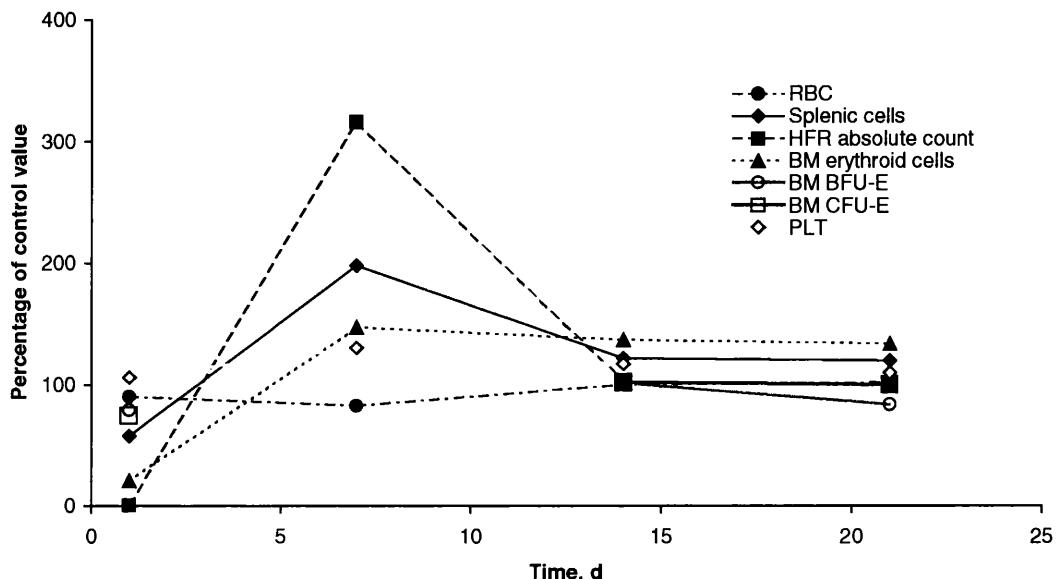


Figure 7.3: Response of erythroid elements and platelets in mice receiving 5 doses of CAPS at 2500 mg/kg/d and sampled at d 1, 7, 14, and 21 post dosing.

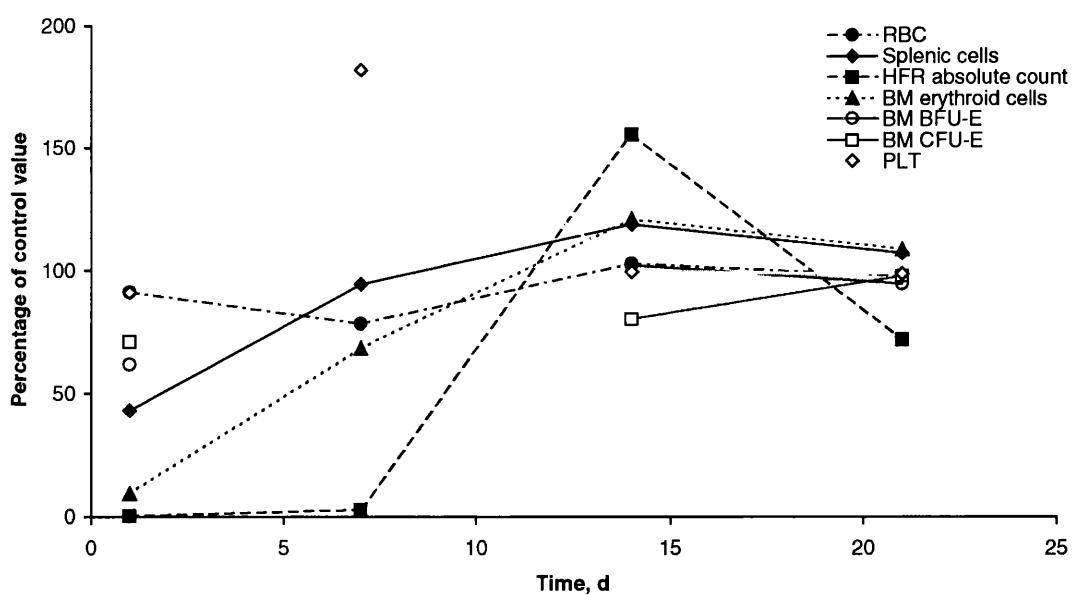


Figure 7.4: Response of erythroid elements and platelets in mice receiving 5 doses of CAPS at 3500 mg/kg/d and sampled at d 1, 7, 14, and 21 post dosing.

At 3500 mg/kg at d 7, highly significant depressions of reticulocytes (total and HFR)

were still evident (Table 7.1; Fig. 7.4). Similarly, many other erythrocyte parameters,

including Hb, RBC and HCT were still significantly reduced at this time. Spleen nucleated cell counts were comparable to the control value at d 7 (Table 7.1; Fig. 7.4), but splenic erythropoietic activity was slight or moderate (Fig. 7.2).

At d 14, most erythrocyte parameters had returned to normal in the 2500 mg CAPS/kg group, but at 3500 mg/kg, there was evidence of regeneration (Table 7.1; Fig. 7.4).

Splenic nucleated cell count, total and HFR reticulocytes showed elevations to 118.7 %, 141.6 % and 155.5 % of the corresponding control values, respectively, but these increases were not statistically significant. However, at 2500 and 3500 mg CAPS/kg, RDW and HDW were significantly increased. By d 21, RDW and HDW were the only two measurements still showing significant elevations.

Platelet counts were significantly elevated at 2500 and 3500 mg CAPS/kg on d 7 (Table 7.1; Fig. 7.3, 7.4), and in animals receiving 3500 CAPS/kg, counts were more than 80 % higher than controls. At all other times, platelet counts were not significantly different from the control value in either dose group.

7.3.3 Haematology: Leucocytes

At 2500 mg CAPS/kg, the total leucocyte count was depressed significantly ($p<0.01$) at d 1 post dosing, as was the CFU-GM count ($p<0.05$; Table 24). The femoral myeloid count on this occasion did not differ from the control value. Numbers of “other” femoral cells, (especially monocytes and monocytic precursors), as well as peripheral LUC counts, were elevated at d 1 in the 2500 mg/kg group, but this elevation was not significant (Table 7.2;

Fig 7.5). Peripheral neutrophils, monocytes, eosinophils and basophils all showed reductions at d 1 in this group, but the reductions were not significant. At 7 d post dosing in the 2500 mg CAPS/kg group, there were significant elevations of LUCs and basophils; “other” femoral cells and monocytes were increased, but not significantly. At d 14 and d 21 post dosing, all leucocyte parameters in the 2500 mg/kg group were close to the corresponding control values (Table 7.2; Fig. 7.5).

At d 1 post dosing in the 3500 mg CAPS/kg group, significant reductions were present in the total leucocyte, neutrophil, lymphocyte, eosinophil and CFU-GM counts (Table 7.2; Fig. 7.6). Eosinophils, basophils and LUC were also reduced, but not significantly. Femoral myeloid cells, however, were slightly, but not significantly, increased, but “other” femoral cells were increased to nearly 300 % of the control value. This increase was significant ($p<0.05$). At d 7, all leucocyte parameters except the basophil count were elevated in the 3500 mg/kg group, however, only the elevation of the LUC count achieved significance. The “other” femoral cell count was still elevated to more than 250 % of the control value on this occasion. At d 14 and 21 post dosing in the 3500 mg CAPS/kg group, most leucocyte parameters were close to their corresponding control values, but femoral myeloid cells and LUC counts in this group were slightly reduced, at 59.1 % to 75.0 % of the corresponding control value; these reductions were not statistically significant (Table 7.2; Fig. 7.6).

In the marrow smears, at both dose levels on d 1, many myeloblasts, promyelocytes, myelocytes and monocyte precursors contained a few small cytoplasmic vacuoles. Furthermore, bone marrow taken from the animal killed on the first day of dosing showed marked vacuolation of myeloid precursors (Fig. 7.1 (a)).

Table 7.2: Mean leucocyte parameters following five consecutive daily CAPS gavage doses at 2500 and 3500 mg/kg. n=5 except at 3500 mg CAPS/kg on d 18 where n=4; for CFU-GM, n=3; *significantly different to control value, p<0.05; **p<0.01; nd = not determined.

CAPS dose, mg/kg	Control				2500				3500			
	1	7	14	21	1	7	14	21	1	7	14	21
Day post dosing	1	7	14	21	1	7	14	21	1	7	14	21
WBC, $\times 10^9/L$	4.43	4.54	4.78	5.80	3.60**	6.44	5.60	5.80	2.60**	5.66	4.35	6.06
Neut, $\times 10^9/L$	0.510	0.530	0.563	0.652	0.265	0.643	0.754	0.660	0.116**	0.708	0.640	0.690
Lymph, $\times 10^9/L$	3.635	3.704	3.862	4.762	3.158	4.884	4.388	4.843	2.374*	4.000	3.416	5.012
Mono, $\times 10^9/L$	0.108	0.125	0.075	0.120	0.048	0.295	0.107*	0.097	0.078	0.244	0.088	0.107
Eos, $\times 10^9/L$	0.100	0.080	0.100	0.140	0.065	0.084	0.070	0.100	0.040*	0.109	0.120	0.140
Baso, $\times 10^9/L$	0.010	0.020	0.010	0.020	0.000	0.090*	0.020	0.020	0.000	0.020	0.010	0.020
LUC, $\times 10^9/L$	0.028	0.024	0.078	0.064	0.065	0.070*	0.070	0.060	0.010	0.036*	0.053	0.048
BM myeloid cells, $\times 10^7$	0.440	0.478	0.433	0.404	0.426	0.506	0.380	0.460	0.598	0.608	0.363	0.604
BM lymphoid cells, $\times 10^7$	0.403	0.378	0.323	0.360	0.280	0.472	0.323	0.498	0.315	0.274	0.288	0.422
Other BM cells, $\times 10^7$	0.035	0.022	0.045	0.044	0.084	0.052	0.028	0.046	0.100*	0.058	0.028	0.026
CFU-GM per plate	28.7	nd	30.3	30.7	19.0*	nd	27.3	29.0	14.3*	nd	26.7	35.3

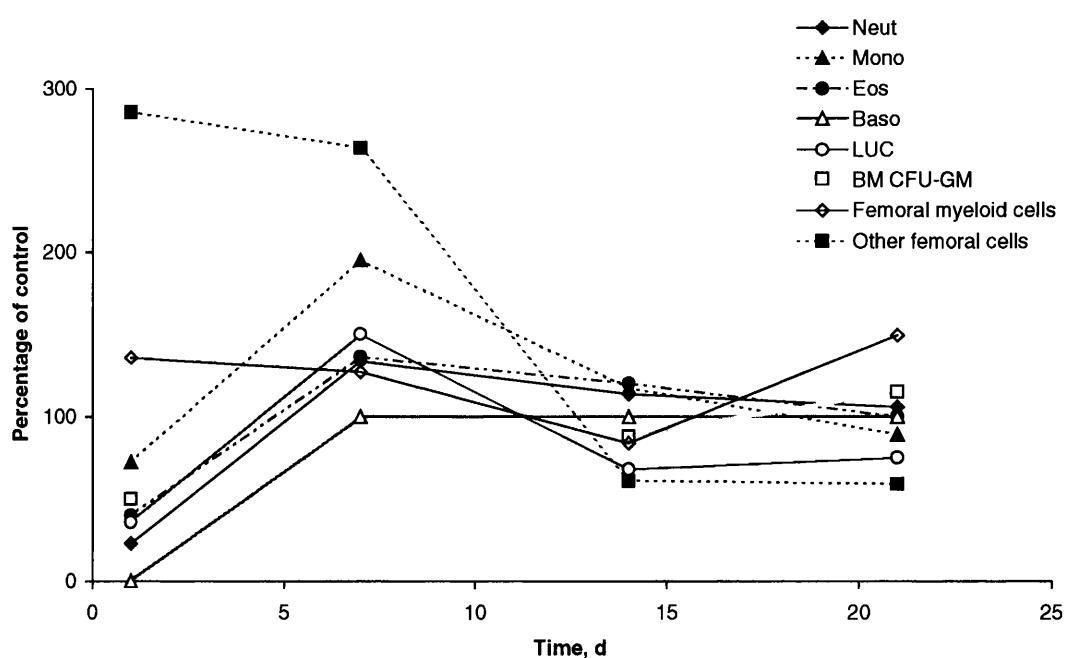
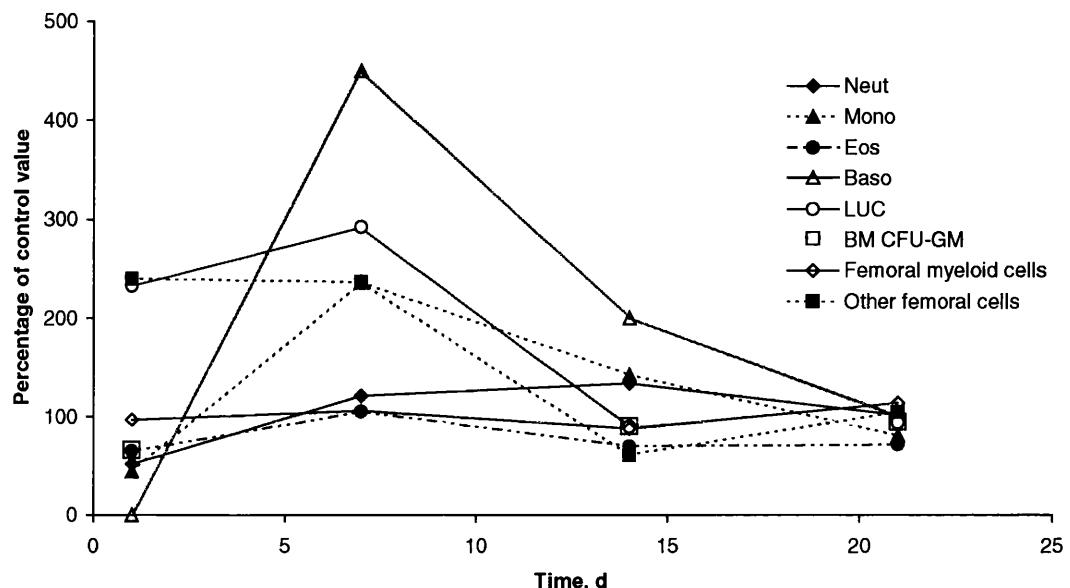


Figure 7.5: Response of myeloid (leucocyte) elements in mice receiving 5 daily doses of CAPS at 2500 mg/kg/d and sampled at d 1, 7, 14, and 21 post dosing.

7.4

Discussion

Using a high level dosing regime at 2500 and 3500 mg CAPS/kg, with the drug administered on five consecutive days, haemotoxicity was demonstrated in the B6C3F₁ mouse. Cessation of erythropoiesis was observed at both dose levels immediately after the dosing period (d 1 post dosing), with recovery seen at 7 d post dosing at 2500 mg CAPS/kg, and between 7 and 14 d post dosing with 3500 mg/kg. Although toxicity was most severe in the erythroid series (Table 7.1; Fig. 7.3, 7.4) at both dose levels, this effect was not specific, as reductions in CFU-GM and peripheral leucocyte counts were also seen at both dose levels (Table 7.2; Fig. 7.5, 7.6). In addition, vacuolation was noted in precursors of both the myeloid and the erythroid series (Fig. 7.1). Whilst femoral CFU-E and BFU-E were depressed immediately after dosing, the myelosuppressive effect was more clearly seen in the morphologically recognisable committed precursor cells in the marrow, and also in the spleen cellularity.

However, the depression was most markedly evident in the HFR reticulocyte fraction. HFR was also the most sensitive measure of erythroid recovery, showing the maximal amplitude of response as erythropoiesis returned to normal. The pattern of erythroid suppression and recovery seen in Experiment 10 is comparable to the reversible toxicity to cells of the erythroid series reported in patients and healthy volunteers (Volini *et al.*, 1950; Saidi *et al.*, 1961; Jiji *et al.*, 1963; Scott *et al.*, 1965). Maturation arrest and vacuolation at the level of the pronormoblast and early normoblast in this experiment is consistent with findings in man (Rosenbach *et al.*, 1960; Scott *et al.*, 1965; Yunis *et al.*,

1970; Skinnider and Ghadially, 1976), rats (Schober *et al.*, 1972) and mice (Miura *et al.*, 1980).

In other studies, for example with methotrexate, a clear relationship has been demonstrated between HFR count and the microscopic assessment of splenic erythropoiesis (Andrews, 1995). However, as demonstrated earlier (Experiment 3, Fig. 3.11, 3.14), the HFR response lags behind that of the spleen and marrow by about one day. This is also illustrated in the present study (Fig. 7.2) by the finding that at d 7, in mice receiving 3500 mg CAPS/kg that there was slight or moderate erythropoietic activity in spleen touch preparations, yet HFR values were still exceptionally low, at 0 - 3.5 (mean 1.48) $\times 10^9/L$ (Table 7.1). At d 7 post dosing, HFR and spleen cellularity at 2500 mg CAPS/kg are seen to exhibit a rebound above control levels to a much greater degree than femoral erythroid cells (Table 7.1; Fig. 7.3), and at d 14 post dosing at 3500 mg CAPS/kg, the HFR rebound occurs in spite of normal levels of femoral BFU-E and depressed CFU-E (Table 7.1; Fig. 7.4). Havard *et al.* (1999a) and Turton *et al.* (1999; 2000) have demonstrated that rebound reticulocytosis can occur in the mouse in spite of continued CAPS dosing and in the presence of depressed femoral CFU-E counts. This finding is in agreement with that of Rigdon *et al.* (1954) and Havard *et al.* (1999b), where reticulocyte rebound occurred in ducks and guinea-pigs, respectively, despite continued administration of CAP. Analysis of reticulocyte morphology during the rebound phenomenon by Rigdon *et al* (1954) demonstrated a response by the most immature forms comparable to the response shown by HFR reticulocytes in the present study.

CAPS has been reported to induce apoptosis in femoral erythroid and myeloid precursors (Holt *et al.*, 1998). This therefore suggests that the spleen may be the site of the

compensatory erythropoiesis. In mice which developed marrow aplasia following the administration of ^{89}Sr (Klassen *et al.*, 1972), normal levels of reticulocyte production and ^{59}Fe incorporation were maintained by splenic erythropoiesis, whilst splenectomised animals died with pancytopenia. Likewise, the administration of estradiol benzoate to mice caused reductions in ^{59}Fe uptake in the femur with markedly increased levels in the spleen, which maintained a normal haematocrit and red cell mass (Anagnostou *et al.*, 1976). In mice treated with thiamphenicol (Goris *et al.*, 1990) and human granulocyte colony-stimulating factor (de Haan *et al.*, 1992), suppression of femoral BFU-E and CFU-E was accompanied by peripheralisation and stimulation of splenic erythroid progenitors, which was interpreted as a net migration of erythroid precursor cells from the marrow to the spleen.

The increase in platelet count at 2500 and 3500 mg CAPS/kg on d 7 (Table 7.1; Fig. 7.3, 7.4) is consistent with EPO-induced thrombocytosis in association with CAP-mediated inhibition of erythrocyte maturation (Jackson *et al.*, 1974). A similar effect on platelet counts in the CAPS-treated mouse has been noted by Turton *et al.* (2000).

Peripheral leucocyte counts returned to normal at d 7 post dosing at 2500 and 3500 mg CAPS/kg compared with erythrocyte counts, which did not return to normal until 14 d post dosing (Tables 7.1 and 7.2), reflecting the lower specificity of CAPS toxicity for cells of the myeloid series and the shorter maturation time of mouse leucocytes (Filmanowicz and Gurney, 1960; Lee *et al.*, 1979). Although CFU-GM were reduced, numbers of recognisable myeloid precursors in the marrow were not decreased. Indeed, monocytes and their precursors were present at greatly increased levels in marrow smears at both dose levels on d 1 dosing; this was followed 7 d later by increased peripheral

monocyte and LUC counts (Table 7.2; Fig. 7.5, 7.6). Neutrophilia following CAP administration has been observed in chronic neutropenia man (Adams and Pearson, 1983), and *in vitro* stimulation of human CFU-GM levels has been demonstrated with sub-therapeutic concentrations of CAP (Bostrom *et al.*, 1986), a finding which has been reproduced *in vitro* in the mouse (R. Fagg, personal communication). Elevated neutrophil counts together with reductions in CFU-GM numbers were seen in BALB/c mice immediately after dosing with CAPS at 2000 mg/kg for 17 d (Turton *et al.*, 2000).

All blood and marrow counts were essentially normal 14 d after dosing at 2500 and 3500 mg CAPS/kg, although sensitive indices of erythrocyte volume and intracellular haemoglobin distribution (RDW, HDW) remained abnormal at 21 d after dosing, reflecting the earlier perturbation of erythropoiesis (Table 7.1). It was therefore considered that, in a future experiment, dosing with CAPS at an intermediate level (3000 mg/kg) should be well tolerated. However it might be expected that CAPS would cause cumulative toxicity if administered as repeated 5-day pulses separated by periods of 16 d.

CHAPTER 8: THE HAEMOTOXICITY OF BUSULPHAN AND HIGH DOSE CHLORAMPHENICOL SUCCINATE IN THE B6C3F₁ MOUSE

8.1 Introduction

The attempt to induce AA in the mouse by combining BU and CAPS treatment (Experiment 6) was unsuccessful. Whereas chronic marrow hypoplasia was seen when BU was given fortnightly at 33 and 40 mg/kg, CAPS, when administered in drinking water at 4.0 mg/mL, did not influence the development of BU-induced aplasia or lymphoma. The preceding studies (Experiments 7-9) demonstrated that CAPS did not lead to significant marrow exposure when administered at low dose levels. The previous study (Experiment 10) also provided evidence that oral administration of the drug by gavage at 2500 and 3500 mg/kg induced significant but reversible myelotoxicity.

The following experiment was designed to investigate whether pre-existing marrow damage induced by BU was necessary for an agent such as CAPS, which usually causes reversible myelotoxicity, to precipitate irreversible AA. This idea had previously been examined by Morley *et al.*, (1976b). In Experiment 6, a dose-dependent persistent suppressive effect on haemopoiesis, maximal at 40 mg/kg, was demonstrated with BU in B6C3F₁ mice. Animals treated on four fortnightly occasions at this dose level and left for a period of six weeks, were subsequently clinically well, but exhibited features of residual impairment of haemopoiesis which included depressed leucocyte, platelet and splenic nucleated cell counts (Andrews *et al.*, 1997, 1998).

In early surveys of CAP-associated AA in man, it was reported that AA did not occur after a single administration of the antibiotic (Wallerstein *et al.*, 1969), and that

intermittent dosing might be necessary for AA induction (Yunis and Bloomberg, 1964). It was concluded that, by combining the BU regimen of Experiment 6 with repeated “pulses” of CAPS administered at 3000 mg/kg/d for 5 d, followed by a recovery period of 16 d, a maximal effect on haemopoiesis could be exerted. Under these circumstances, an increase of AA cases in BU+CAPS-treated animals, in comparison with animals dosed only with BU, would provide support for the proposition that the idiosyncratic sensitivity to CAP in man is underlain by residual marrow damage. To further characterise such damage, and the involvement of CAPS in the injury, progenitor and stem cell assays would be included in the parameters assessed.

8.2 Materials and Methods

Mice were obtained from Harlan UK (Bicester, Oxon). Blood, bone marrow and spleen sampling and analysis were as described in Chapter 2 (tibial marrow was taken for smears for microscopic examination). Bone marrow stem cell analysis and progenitor cell assays were performed as described in Chapter 2.

8.2.1 Experiment 11: 252-day Study with Busulphan Administration and Intermittent High Dose Chloramphenicol Succinate

Two groups of 26 (groups 1 and 2) and two groups of 41 (groups 3 and 4) mice received the following treatments: group 1, controls; group 2, CAPS; group 3, BU; group 4, BU + CAPS (Table 8.1). Animals in groups 3 and 4 received ip 40 mg BU/kg on four fortnightly occasions (d 0, 15, 29 and 42). Animals in groups 2 and 4 received CAPS by gavage at 3000 mg/kg for 5 consecutive d for eight dosing cycles, starting 42 d after the last BU dose (i.e. CAPS dosing starting on d 84, 105, 126, 147, 168, 189, 211 and 231). Each CAPS dosing cycle consisted of a “pulse” of 5 daily doses of CAPS, followed by a

recovery period of 16 d. At the end of the second, fourth, sixth and eighth CAPS dosing cycles (i.e on d 126, 168, 211 and 252), 5 animals per treatment group were killed and blood, marrow and spleen taken for full blood count, differential leucocyte count, reticulocyte count, femoral nucleated cell count, femoral stem cell ($CD117^+$) count, femoral progenitor cell assays (BFU-E, CFU-E and CFU-GM), and splenic nucleated cell counts. Animals which were found to be unwell or which had marked weight loss were killed (ICD); where possible, the same samples were taken from these ICD animals. Body weights were recorded weekly.

Table 8.1: Design of Experiment 11. B, M, S = samples taken for full blood count, marrow and spleen cell counts.

Group	n	Day		
		0, 15, 29, 42 BU, 40 mg/kg	84 onwards CAPS, 3000 mg/kg	126, 168, 211, 252 5 mice per group
1	26			B, M, S
2	26		8 × (5 daily doses + 16 d recovery)	B, M, S
3	41	✓		B, M, S
4	41	✓	8 × (5 daily doses + 16 d recovery)	B, M, S

8.3 Results

8.3.1 Survival

One BU-treated mouse in group 3 died on d 29, the day of administration of the third BU dose. In the period immediately following BU dosing (i.e., after d 42), in groups 3 (BU) and 4 (BU + CAPS) there was an unexpectedly high incidence of ICD especially up to d 60, which then tailed off up to d 80 and beyond (Fig. 8.1). Survival by d 126, the first sampling occasion, in the two BU-treated groups was 37.8 % (33 surviving, and 49 not surviving, out of an initial total of 82 animals). All ICD animals killed up to d 156 had

marked weight loss, pale extremities (ears, tail and paws), had lost condition and appeared hunched and inactive.

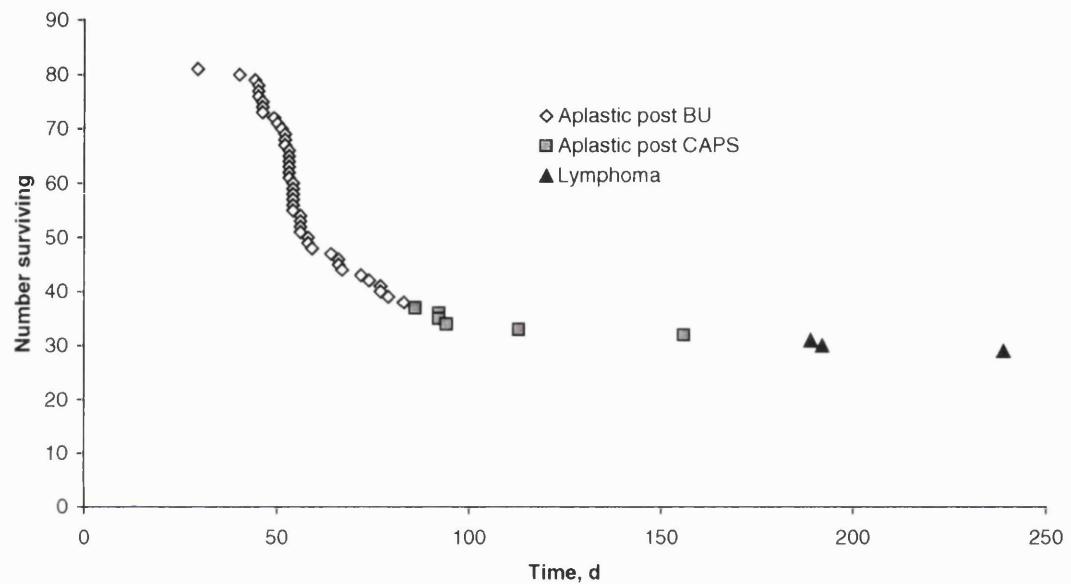


Figure 8.1: Survival in BU-treated animals (groups 3 and 4 combined, initial n=82).
BU (40 mg/kg) was administered on d 0, 15, 29 and 42; CAPS (3000 mg/kg/d for 5 d) was administered to group 4 in 21 d cycles from d 84 onwards.

8.3.1.1 Analysis of the Influence of Chloramphenicol Succinate Administration on Intercurrent Deaths

Forty-four mice died up to d 84. Of nine ICD animals killed, due to loss of condition, after the start of the first CAPS administration on d 84, almost equal numbers were from group 3 (total 5), which had received BU alone, and from group 4 (total 4), receiving BU and CAPS (Table 8.2). In addition, six mice of the nine that showed loss of condition, and which were also found to be aplastic (ICD on d 86, 92 (2), 94, 113 and 156) were equally distributed between the two treatment groups. Three mice were found to have developed lymphoma (ICD on d 189, 192 and 239; Tables 8.2 and 8.4) and of these, two had received BU alone (group 3) and one, BU and CAPS (group 4). No mortality was seen in the vehicle treated controls (group 1) or in group 2 (CAPS alone).

Table 8.2: Incidence of intercurrent deaths in BU-treated mice (groups 3 and 4) after the start of CAPS administration (d 84). A, indicates aplastic animal; L, presence of lymphoma.

Day	Group 3 BU alone	Group 4 BU + CAPS
86	A	
92	A	
92		A
94		A
113		A
156	A	
189		L
192	L	
239	L	
Total aplastic	3	3
Total lymphoma	2	1

8.3.1.2 Intercurrent Deaths to day 156: Haematology

There were 50 ICD mice from the first day of BU administration until d 156. In these 50 animals, peripheral cell counts showed a marked reduction in most cell types (Table 8.3). Compared with mice sampled at the first scheduled *post mortem* on d 126, the mean neutrophil count of the 50 ICD animals was 7.3 % of the mean control value. The corresponding values for lymphocytes, monocytes and eosinophils were 32.4 %, 15.3 % and 2.1 %, respectively. The recorded basophil and LUC counts in the ICD mice were not different to the control values. The mean platelet count of the ICD mice was 3.3 % of the d 126 control mean, haemoglobin was 18.3%, total reticulocyte count 21.9 % and HFR count 30.0 %, respectively. The mean cell volume was significantly increased, with slight to marked anisocytosis and slight to moderate macrocytosis being flagged in 40/50 and 42/50 animals, respectively. The cellularity of bone marrow and spleen were markedly reduced, with the numbers of femoral haemopoietic cells reduced to 5.7 % of the mean d 126 control value. The mean spleen weight in the ICD mice was reduced to 67.8 % of d 126 control mean value ($p<0.05$); the reduction in spleen cell count was to 36.0 % of the d 126 control mean ($p<0.001$).

Table 8.3: Mean (sd) of peripheral cell counts, femoral and spleen nucleated counts and spleen weight in ICD mice dying between d 29 and 156 (n=50). BU (40 mg/kg) was administered to 82 animals (groups 3 and 4) on d 0, 15, 29 and 42. Mean values of control mice killed at scheduled times on d 126 and 168 are shown for comparison, n=5. *p <0.05; *p <0.001 vs d 126 controls.**

Group	Day	Hb g/dL	MCV fL	Plt x10 ⁹ /L	Retic x10 ⁹ /L	HFR %	HFR x10 ⁹ /L
ICD	29-156	2.66 (1.35)***	62.99 (6.90)***	39.1(36.7)***	64.64 (91.42)***	20.44 (9.52)	13.04 (16.70)***
Control	126	14.50 (0.23)	52.76 (1.00)	1171.0 (85.3)	294.68 (86.40)	15.90 (4.08)	48.36 (24.53)
Control	168	14.46 (0.42)	52.74 (0.68)	1111.0 (78.5)	279.92 (32.15)	10.56 (1.82)	29.57 (6.00)

Group	Day	WBC x10 ⁹ /L	Neut x10 ⁹ /L	Lymph x10 ⁹ /L	Mono x10 ⁹ /L	Eos x10 ⁹ /L	Baso x10 ⁹ /L	LUC x10 ⁹ /L
ICD	29-156	1.26 (0.43)***	0.043 (0.088)***	1.130 (0.417)***	0.015 (0.051)***	0.002 (0.004) ***	0.018 (0.015)	0.050 (0.043)
Control	126	4.36 (0.35)	0.588 (0.216)	3.482 (0.201)	0.098 (0.018)	0.088 (0.036)	0.018 (0.013)	0.066 (0.048)
Control	168	3.64 (0.65)	0.536 (0.107)	2.794 (0.516)	0.078 (0.024)	0.144 (0.050)	0.010 (0.000)	0.070 (0.034)

Group	Day	BM count x10 ⁷	Spleen weight g	Splenic cells x10 ⁷
ICD	29-156	0.132 (0.483)***	0.0618 (0.0251)*	6.428 (2.386)***
Control	126	2.324 (0.830)	0.0912 (0.0079)	17.840 (1.098)
Control	168	1.808 (0.318)	0.0826 (0.0105)	17.078 (4.509)

Table 8.4: Peripheral cell counts, femoral and spleen nucleated counts and spleen weight in three ICD mice dying on d 189, 192 and 239. The animal on d 239 was found dead and blood and femoral marrow samples could not be obtained. BU (40 mg/kg) was administered on d 0, 15, 29 and 42. Mean values (sd) of control mice on d 168 and 252 are shown for comparison; n=5.

Group	Day	Hb g/dL	MCV fL	PLT x10 ⁹ /L	Retic x10 ⁹ /L	HFR %	HFR x10 ⁹ /L
ICD	189	12.0	49.2	838	229.9	12.9	29.7
ICD	192	3.1	51.1	15	33.2	11.9	4.0
ICD	239						
Control	168	14.46 (0.42)	52.74 (0.68)	1111.0 (78.5)	279.92 (32.15)	10.56 (1.82)	29.57 (6.00)
Control	252	14.68 (0.36)	52.16 (0.56)	1122.4 (45.7)	273.48 (58.15)	17.42 (4.22)	47.59 (16.58)

Group	Day	WBC x10 ⁹ /L	Neut x10 ⁹ /L	Lymph x10 ⁹ /L	Mono x10 ⁹ /L	Eos x10 ⁹ /L	Baso x10 ⁹ /L	LUC x10 ⁹ /L
ICD	189	6.15	2.70	2.33	0.13	0.03	0.15	0.82
ICD	192	0.49	0.01	0.42	0.00	0.00	0.03	0.06
ICD	239							
Control	168	3.64 (0.65)	0.536 (0.107)	2.794 (0.516)	0.078 (0.024)	0.144 (0.050)	0.010 (0.000)	0.070 (0.034)
Control	252	5.16 (0.90)	0.710 (0.125)	4.106 (0.718)	0.140 (0.043)	0.134 (0.033)	0.012 (0.004)	0.092 (0.019)

Group	Day	BM count x10 ⁷	Spleen weight g	Splenic cells x10 ⁷
ICD	189	2.0	0.085	10.81
ICD	192	0.4	0.144	6.30
ICD	239	-	0.551	70.31
Control	168	1.808 (0.318)	0.0826 (0.0105)	17.078 (4.509)
Control	252	2.062 (0.487)	0.0984 (0.0130)	15.362 (1.345)

8.3.1.3 Intercurrent Deaths after day 156

Only three ICDs occurred after d 156 (Fig. 8.1; Table 8.4). One died following CAPS dosing on d 189. This animal was slightly anaemic, and the LUC was increased. A few lymphoma cells were present in the blood and bone marrow smears and in the spleen imprint. Other haematology in this animal was relatively normal, and femoral and splenic cellularity was normal (Table 8.4). A second animal was killed due to its poor condition on d 192, and one was found dead on d 239. In each case the clinical condition of these two animals was similar to the earlier ICDs (up to d 156). The haematology of the d 192 ICD mouse showed marked reductions in peripheral cell counts and femoral cellularity (Table 8.4), but the spleen was enlarged, with many lymphoma cells present in the imprint. Lymphoma cells were also present in the blood and marrow smears. No blood sample was obtained from the animal found dead on d 239, but the spleen was greatly enlarged (Table 8.4), with the imprint showing massive lymphoma infiltration. In all three cases, enlargement of the thymus was noted at autopsy.

8.3.2 Scheduled Bleeds: Haematology

Due to the unexpectedly high mortality in BU-treated animals (groups 3 and 4), insufficient numbers remained in the BU and BU + CAPS-treated groups for scheduled bleeds at all four planned timepoints (d 126, 168, 211, 252). The d 211 bleed was therefore not carried out on groups 3 and 4.

8.3.2.1 Effects of Chloramphenicol Succinate (Group 2)

The principal effect of CAPS alone dosing (group 2) was to induce an apparent thrombocytosis, and a microcytosis of erythrocytes without significantly affecting the rate of red cell production (ie reticulocyte count) (Table 8.5 (a); Fig 8.2). Platelet counts were elevated on all occasions, but significantly so at d 168, 211 and 252. MCV was significantly reduced, and percentage microcytes, RDW and HDW were all significantly elevated on each occasion. The mean Hb and HCT were slightly reduced at all timepoints, significantly so at d 252. The mean RBC, however, was slightly increased at all timepoints and this increase was significant ($p<0.01$) at d 211. The absolute reticulocyte count was elevated at d 252, but not significantly, however, the increase in the absolute LFR count on this occasion did achieve significance ($p<0.05$).

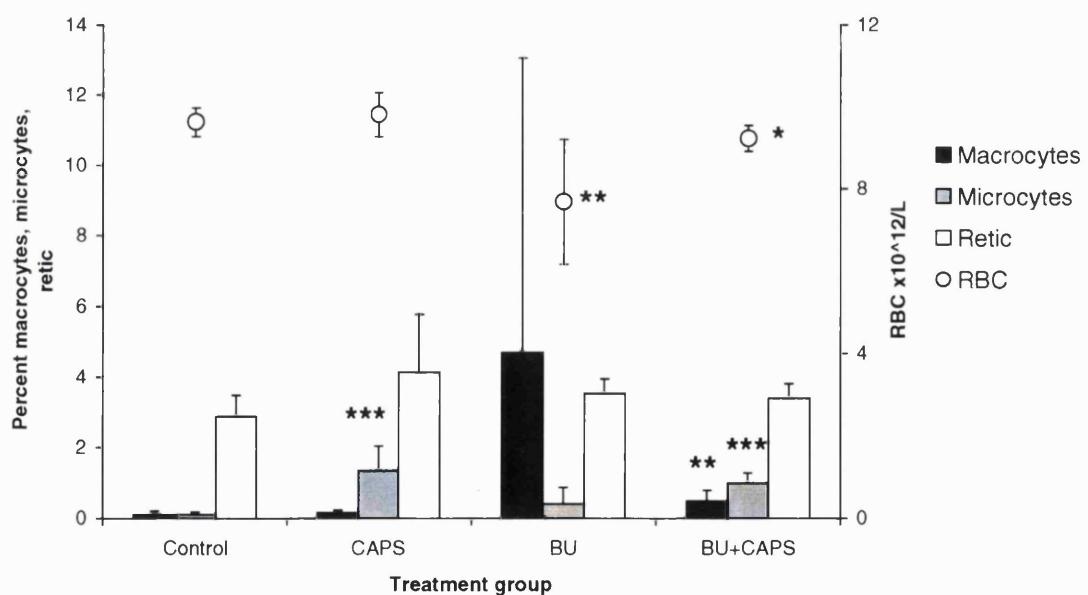


Figure 8.2: Mean and sd of erythrocyte count, percent reticulocytes, macrocytes and microcytes in mice on d 252 following four fortnightly BU doses (40 mg/kg) and intermittent CAPS dosing (3000 mg/kg). n=5; *significantly different to control value, p<0.05; ** p<0.01; *p<0.001.**

Table 8.5: Effect of BU and CAPS on (a) erythrocyte measurements and platelet counts; (b) leucocyte counts; (c) femoral bone marrow counts, stem cell counts, and colony-forming unit assays, spleen counts and spleen weights. BU (40 mg/kg ip) was given on d 0, 15, 29 and 42 and CAPS (3000 mg/kg by gavage) 5 d/wk every fourth week from d 82. Samples were not taken from BU-treated animals (groups 3 and 4) on d 211 due to reduced survival in these groups. nd = not determined. Values are means from 5 animals, except for colony-forming unit assays, where n=3; *significantly different to control value, p<0.05; **p<0.01; *p<0.001.**

Table 8.5 (a)

Group	Day	RBC x 10 ¹² /L	Hb g/dL	HCT L/L	MCV fL	MCH pg	MCHC g/dL	RDW %	HDW g/dL	Retic x 10 ⁹ /L	LFR x 10 ⁹ /L	MFR x 10 ⁹ /L	HFR x 10 ⁹ /L	PLT x10 ⁹ /L	
1	Control	126	9.640	14.50	0.509	52.76	15.06	28.50	11.78	1.644	294.68	169.51	76.80	48.36	1171.0
1	Control	168	9.690	14.46	0.511	52.74	14.94	28.28	11.90	1.626	279.92	185.35	65.00	29.57	1111.0
1	Control	211	9.664	14.52	0.491	50.80	15.02	29.58	11.72	1.590	297.06	159.40	87.62	50.05	1146.0
1	Control	252	9.762	14.68	0.511	52.16	15.06	28.74	11.60	1.560	273.48	145.70	80.19	47.59	1122.4
2	CAPS	126	9.672	14.08	0.491	50.74**	14.56*	28.66	14.44***	1.872**	331.90	208.70	79.35	43.85	1238.0
2	CAPS	168	9.776	13.80	0.497	50.80**	14.10**	27.76*	15.66***	1.804***	302.84	172.07	77.36	53.41*	1242.8*
2	CAPS	211	10.266**	14.46	0.497	48.44***	14.10***	29.08*	14.72***	1.720**	246.24	147.70	60.28	38.26	1395.6**
2	CAPS	252	9.788	14.10*	0.479**	49.02***	14.40**	29.38**	16.20***	1.854***	400.10	231.30*	107.03	61.77	1487.8**
3	BU	126	8.902	13.64	0.477	53.64	15.36	28.60	12.50*	1.618	312.48	159.36	96.03	57.08	666.4**
3	BU	168	8.786*	13.48	0.478	54.54	15.36	28.14	12.80*	1.644	294.20	146.84*	84.99*	62.38**	566.8**
3	BU	252	7.675**	12.18*	0.418**	55.13	16.05*	29.15*	14.13	1.665	261.43	137.69	71.62	52.11	809.8*
4	BU + CAPS	126	7.428	11.24	0.393	52.36	15.38	29.42	15.50*	1.820**	212.60	111.50	57.87	43.23	401.6**
4	BU + CAPS	168	9.183	13.13*	0.479*	52.25	14.30**	27.35**	16.05***	1.770**	317.28	155.12	90.42*	71.74***	886.0*
4	BU + CAPS	252	9.218*	13.42**	0.468**	50.78	14.54	28.62	15.82***	1.726***	310.53	159.99	84.27	66.27	1574.6***

Table 8.5 (b)

Group	Day	WBC x10 ⁹ /L	Neut x10 ⁹ /L	Lymph x10 ⁹ /L	Mono x10 ⁹ /L	Eos x10 ⁹ /L	Baso x10 ⁹ /L	LUC x10 ⁹ /L
1 Control	126	4.36	0.588	3.482	0.098	0.088	0.018	0.066
1 Control	168	3.64	0.536	2.794	0.078	0.144	0.010	0.070
1 Control	211	5.02	0.588	4.134	0.152	0.106	0.012	0.040
1 Control	252	5.16	0.710	4.106	0.140	0.134	0.012	0.092
2 CAPS	126	5.10	0.742	4.062	0.114	0.096	0.012	0.056
2 CAPS	168	4.36	0.818	3.280	0.086	0.078*	0.010	0.072
2 CAPS	211	3.30*	0.426	2.714*	0.094*	0.054	0.012	0.022
2 CAPS	252	6.94	1.766*	4.760	0.198	0.102	0.020	0.080
3 BU	126	1.96***	0.366	1.432***	0.080	0.038	0.006	0.026
3 BU	168	1.67***	0.246**	1.306***	0.048	0.028**	0.006	0.032
3 BU	252	2.68**	0.443*	2.028**	0.078*	0.043**	0.005	0.040*
4 BU + CAPS	126	2.02**	0.628	1.198***	0.120	0.032	0.004	0.028
4 BU + CAPS	168	1.93*	0.395	1.395*	0.035*	0.038*	0.008	0.035
4 BU + CAPS	252	5.24	2.020*	2.832*	0.190	0.118	0.014	0.066

Table 8.5 (c)

Group	Day	BM x 10 ⁷	BM		BM		BM		Spleen wt g	Splenic cells x 10 ⁷
			CD117+ x 10 ⁷	CFU-GM /femur	CFU-E /femur	BFU-E /femur	Myeloid x 10 ⁷	Erythroid x 10 ⁷		
1 Control	126	2.324	0.099	nd	nd	0.817	0.702	0.738	0.0912	17.840
1 Control	168	1.808	0.099	29.7	56.7	nd	0.649	0.560	0.564	0.0826
1 Control	211	2.552	0.150	28.6	55.0	nd	0.945	0.772	0.815	0.0964
1 Control	252	2.062	0.073	33.7	51.0	51.3	0.740	0.678	0.566	0.0984
2 CAPS	126	2.778	0.129	nd	nd	nd	0.998	0.938	0.807	0.0942
2 CAPS	168	2.338	0.115	27.3	53.0	nd	0.903	0.769	0.592	0.0980
2 CAPS	211	2.388	0.114	25.2	49.8	nd	1.079	0.657	0.624	0.0922
2 CAPS	252	2.404	0.078*	32.7	54.0	49.3	1.086	0.583	0.574	0.1108
3 BU	126	1.642	0.081	nd	nd	nd	0.511	0.711	0.390	0.0758**
3 BU	168	1.174*	0.078	21.7*	44.3**	nd	0.570	0.376	0.323*	0.0862
3 BU	252	0.596**	0.025*	30.0	49.0	51.0	0.155**	0.237*	0.171**	0.0878
4 BU + CAPS	126	1.910	0.153	nd	nd	nd	0.784	0.707	0.382*	0.0672***
4 BU + CAPS	168	1.048**	0.064	26.0	50.3*	nd	0.317*	0.457	0.240**	0.0696
4 BU + CAPS	252	2.023	0.051	34.0	55.0	51.0	0.934	0.612	0.443	0.0902
										12.200*

Results for leucocytes showed that lymphocyte count was significantly reduced on d 211 (Table 8.5 (b)). On d 252, neutrophil counts were significantly increased.

Bone marrow cellularity was not significantly different from the control value on any occasion, however, the number of CD117⁺ positive cells in the marrow was decreased on d 252, and this increase was significant ($p<0.05$; Table 8.5 (c)). Femoral CFU-GM, CFU-E and BFU-E and morphologically identifiable erythroid and lymphoid cells were not significantly different from the control values on any occasion, however, the number of femoral myeloid cells was increased, but not significantly, at each occasion (114.1 to 146.8 % of controls).

Spleen weight showed slight increases on d 168 and 252, but these increases were not significant (Table 8.5 (c)). Splenic cellularity, on the other hand, was increased on all occasions, and on d 252, where the mean count in this group was 124.4 % of the control value, this increase was significant ($p<0.05$).

8.3.2.2 Effects of Busulphan (Group 3)

The administration of BU at 40 mg/kg on d 0, 15, 29 and 42 to mice in group 3 induced a slight reduction in Hb, RBC and HCT on d 126, 168 and 252, and on d 252 these reductions were statistically significant (Table 8.5 (a); Fig 8.2). MCV was elevated, but not significantly, on all occasions. The percentage of macrocytic cells was significantly increased at each timepoint, as was the RDW, except for d 252. There was no significant effect on the total reticulocyte count on any occasion, although the total HFR count was elevated on d 168; however, all treatment groups (groups 2, 3 and 4)

showed a significant elevation against the control count on this occasion. Platelets were significantly reduced on all occasions (Table 8.5 (a)).

Leucocyte counts showed highly significantly reductions at all timepoints (Table 8.5 (b)). Most leucocyte types were affected, but the most consistent effect was the reduction in lymphocytes.

Femoral marrow cellularity was reduced on each occasion and on d 168 and 252, the reductions were significant (Table 8.5 (c)). On d 168, Femoral CFU-GM and CFU-E were significantly reduced, together with the femoral lymphoid count (Table 8.5 (c)). On d 252 the total number of femoral CD117⁺ cells as well as morphologically identifiable myeloid, erythroid and lymphoid cells all showed significant reductions.

Spleen weight and cellularity showed highly significant reductions at d 126 (Table 8.5 (c)), and on d 252, spleen cellularity was again significantly reduced, but spleen weight was not different to the control value on this occasion.

8.3.2.3 Combined Effects of Busulphan and Chloramphenicol Succinate (Group 4)

The haematological changes in this group appeared to reflect only the residual effects of both treatments; there was no evidence that the combined administration of both drugs at high dose levels induced AA. However, the changes induced by dosing with BU and CAPS were inconsistent, especially on d 252 when there was considerable individual variation. Reductions in Hb, RBC and HCT were present on all occasions (Table 8.5 (a)), but significant changes were only seen in all three measurements at d 252. Reticulocytes were unaffected at d 126, 168 and 252 except for MFR and HFR at d 168 and there was

slight macrocytosis at d 252 (Fig. 8.2), although the MCV was not significantly different to the control value at any timepoint. Platelets were significantly reduced on d 126 and 168, but increased on d 252 (Table 8.5 (a)).

Lymphocytes were significantly reduced on d 126, 168 and 252; on d 126 this reduction was highly significant (Table 8.5 (b)).

Femoral cellularity, myeloid cells and CFU-E were reduced on d 168 alone, but the absolute number of femoral lymphoid cells were reduced on all occasions, but only significantly so on d 126 and 168 (Table 8.5 (c)). Spleen cellularity was reduced on all occasions, but only significantly so on d 126 and 252 (Table 8.5 (c)).

8.4 Discussion

The haemopoietic lesion induced by BU alone in group 3 mice was similar in magnitude, the range of affected parameters, and persistence, to that seen in Experiment 5. However, the attempt to generate aplasia by the administration of BU followed by high-dose CAPS (in group 4) was unsuccessful. Indeed, in ICD animals, in groups 3 and 4, the administration of CAPS at 3000 mg/kg, a dose level which was at least seven times higher than the daily dose given in Experiment 6, had no effect in modifying or enhancing the BU-induced lesion. Similarly, in animals in group 4 sampled at the scheduled times on d 126, 168 and 252, the effect of CAPS dosing had little added effect on the BU-alone induced changes (Table 8.5; Fig. 8.2). These findings contrast with those of Morley *et al.* (1976b), where progressive reductions in CFU-S and CFU-GM were seen in mice given BU and CAPS. In the present experiment, no differences were observed, between mice

receiving BU and CAPS, and those receiving BU alone, in marrow cellularity, CD117⁺ cells, CFU-GM, CFU-E, or BFU-E.

In CAPS-treated mice, microcytosis with slight elevations of RBC was present at all scheduled autopsy timepoints (Table 8.5 (a); Fig. 8.2). These observations are in contrast to those in Experiments 4 and 5, where the only changes seen in erythrocyte parameters were due to inappetance and reduced water intake. The present findings are consistent with the inhibition of mitochondrial haem synthesis which has been reported by Manyan *et al.* (1972) and Rosenberg and Marcus (1974) in CAP-treated dogs and rabbits. Mitotic division of maturing erythroid cells is inhibited by full haemoglobinisation. Under conditions of reduced haem (and therefore, haemoglobin) synthesis, extra divisions of intermediate and late normoblasts occur, leading to increased numbers of smaller mature erythrocytes, a so-called "thalassaemic" picture. This change in the erythrocyte population is reflected in an increase in RDW and HDW, as seen in Experiment 10, and the present experiment.

Consistent elevations of the platelet count were also seen in CAPS-treated animals, presumably as a result of EPO-induced stimulation of megakaryopoiesis (Jackson *et al.*, 1974), as was seen in Experiment 10. Alterations to leucocyte counts were generally minor and inconsistent.

It has been reported that the serum concentration of CAP falls during continued CAPS administration in man (Nahata and Powell, 1983). In the present experiment, serum levels of CAP and CAPS were not determined. Nevertheless, the haemotoxic effects on erythropoiesis and platelet production in CAPS-treated animals (group 2; Table 8.5 (a)),

were still present at the scheduled autopsies at d 126, 168, 211 and 252, which were each at 16 d after the last CAPS administration. In Experiment 10, on the other hand, at 14 d post CAPS dosing, the alterations in erythrocyte and platelet measurements, with the exception of RDW and HDW, were absent (Table 7.1). Furthermore, the CAPS-induced erythrocyte and platelet effects in group 2 animals did not decrease with time (Table 8.5 (a)).

The 50 BU-treated ICD animals which became ill and were killed, or were found dead, in the early part of the experiment were identified as having profound cytopenias and markedly decreased femoral and splenic cellularity (Table 8.3). The anaemia seen in these animals was nonregenerative and macrocytic, but peripheral LUC counts were normal. When peripheral blood and bone marrow counts are scaled to account for species differences, the murine values satisfy the criteria for SAA in man (see Chapter 1.1.3). Based on the criteria proposed by Camitta *et al.*, (1976; 1982b), SAA may be diagnosed in the mouse when severe peripheral pancytopenia is present, with granulocytes $<0.23 \times 10^9/L$ (one third of the control mean), platelets $<175 \times 10^9/L$ (15 % of the control mean) and a corrected reticulocyte percentage of $<1\%$. Bone marrow cellularity would be $<0.58 \times 10^7$ (25 % of the control mean). All except one of these 50 ICD animals were profoundly anaemic; granulocytes and femoral marrow counts satisfied the criteria for a murine SAA in all cases. However, in the ICD mice, the corrected reticulocyte count was greater than 1 % in 8/50 cases, reflecting the capacity for compensatory splenic haemopoiesis in the mouse (Filmanowicz and Gurney, 1960; Bozzini *et al.*, 1970; Spivak *et al.*, 1972).

Criteria for murine SAA, therefore, bearing in mind splenic erythropoiesis, cannot be directly extrapolated from criteria for human SAA. In the aplastic ICD mice, the mean splenic nucleated cell count was 36.0 % of the control mean at d 126, but because the

erythroid composition of the mouse spleen is normally only approximately 5 % (Spivak *et al.*, 1972), the total cell count cannot be more than an indirect indication of haemopoietic activity. Nevertheless, for the spleen, the total cell count is more sensitive than weight, which was 67.8 % of the d 126 control mean in the aplastic ICD mice. This discrepancy (i.e., 67.8 % spleen weight versus 36.0 % cell count) may have been due to a greater proportion of the weight of ICD spleens being composed of blood and capsular material. Similar discrepancies have been noted in tumour-bearing rats with haemolytic anaemia (Stromberg *et al.*, 1990).

In animals which had received BU alone (group 3), sampled at d 126, 168 and 252, there were also reductions in RBC, Hb and HCT on d 126 and 168, but these did not all become significant until d 252 (Table 8.5 (a)). This slight anaemia was not fully compensated, and total reticulocyte counts were at no time significantly different from controls. Slight but non-significant elevations of MCV, and increased RDW, significant on d 126 and 168, were present but these changes were not as marked as those seen in the ICD mice. Whilst femoral cellularity and absolute numbers of c-kit⁺ (CD117⁺) cells were consistently reduced in BU-treated mice at the scheduled autopsies, significance was only achieved for both measurements on d 252. Indeed, the percentage of cells positive for CD117 was not different from the control value at any time. Nevertheless, these statistically insignificant reductions in CD117⁺ numbers may be reconciled with the finding of Morley *et al.*, (1976b), whose reported reductions in CFU-S after BU (and CAPS) administration also did not achieve statistical significance on a number of occasions. Numbers of committed precursor cells (CFU-GM, CFU-E) in BU-treated mice showed reductions (Table 8.5 (c)), but these were not uniformly statistically significant. The effect on splenic cellularity was also inconsistent.

As in Experiment 6, the effect of BU in groups 3 and 4 was to reduce peripheral platelet and lymphocyte counts in surviving animals. Indeed, for BU-only treated animals (group 3), these were the the most consistent markers of myelosuppression, but for those animals also receiving CAPS (group 4), these effects were masked at d 252 (Table 8.5 (a) and (b)).

The effect of combined dosing with BU and CAPS was to produce an inconsistent pattern of change reflecting some of the features seen in animals which received either of the two drugs alone. For example, the lack of effect on MCV reflected the combination of CAPS-induced microcytosis and BU-induced macrocytosis. A similar “masking” of macrocytosis may be seen in human AA when co-existing with β -thalassaemia, a condition associated with microcytosis (Alter, 1998). Similarly, in this group, there were reductions and increases in the platelet count and other cell types, rather than any overall additive effect.

A major difference between the present experiment and Experiment 6, which soon became apparent during the progress of the study, was increased mortality from BU administration. In Experiment 6, 50 % of animals dosed at 40 mg BU/kg survived until d 279. In the present experiment, 50 % mortality was reached by d 77. BU in each experiment was obtained from the same supplier, Sigma Chemical Co. Ltd, Poole, Dorset, and was specified to be of similar purity, in each case, greater than 99 %. Whilst it is well known that considerable interstrain differences exist in mice in response to drug treatment, for example, in response to CAPS (Miller *et al.*, 1978; Festing *et al.*, 2000), and in haemopoietic stem cell content (de Haan *et al.*, 1997), mice of the same strain and

possessing identical genetic constitution would be expected to respond to xenobiotics in a similar manner. Animals for Experiments 6 and 11 were of the same strain and age range, but were obtained from within Glaxo Wellcome (Experiment 6) and from an external supplier (Harlan UK, Oxford) for Experiment 11. Confirmation of the genetic identity of the breeding stock from the two sources was sought using microsatellite analysis. This analysis strongly suggested C57/BL6 and C3H parental lineage for both stock lines (Charles River UK, personal communication), indicating genetic similarity.

In attempting to identify a possible basis for the increased mortality in Experiment 11, BU dosing in Experiment 6 took place in February/March (1992), and in June/July (1998) in Experiment 11. It has been observed elsewhere that tumour incidence in mice may change over time. For example, Scales and Andrews (1991) observed a doubling of the incidence of leukaemia in the C57BL mouse, from 40 % to over 80 %, over a ten-year period. Similar findings are reported elsewhere (Haseman *et al.*, 1998) and it is known that factors such as housing conditions and diet, influence the rate of tumour occurrence (Everitt, 1984; Hardisty, 1985; Sheldon *et al.*, 1995). It may be of relevance that seasonal variation in toxicity and mortality in mice has also been reported elsewhere (Sirotnak *et al.*, 1977; Perissin *et al.*, 1998). The LD₅₀ of DDMP, a 5-amylpyridine antifolate, in male BDF₁ mice showed a twofold variation over a two year period, with a lower LD₅₀ seen between April and September and higher values between October and March (Sirotnak *et al.*, 1977). Similarly, rotational-stress mediated metastasis of transplanted Lewis lung carcinoma in mice showed an annual periodicity, with a summer peak corresponding with greater stress-induced reductions in CD3⁺ and CD4⁺ lymphocytes (Perissin *et al.*, 1998).

It is also known that such annual variations in cell proliferation occur in the haemopoietic organs of mice. Laerum *et al.* (1988) reported increased numbers of bone marrow cells in G₂ and S phase in winter months. Haus *et al.* (1997) found a two-fold difference in winter/summer bone marrow ³H thymidine uptake which was independent of day length, with lower values in summer. On this basis, a greater proportion of haemopoietic stem cells would have been out of cell cycle in mid summer when dosing took place in Experiment 11, which would in turn have presented a greater target for BU toxicity. This is in direct contrast to toxicity by agents targetting committed haemopoietic progenitors such as AZT, where increased toxicity has been demonstrated with increasing marrow proliferative activity (Scheding *et al.*, 1994).

CHAPTER 9: FINAL DISCUSSION

9.1 Summary of Findings in the Present Work

9.1.1 Characterisation of Busulphan and Chloramphenicol Succinate Haemotoxicity in the B6C3F₁ Mouse

The MTSD for BU in the B6C3F₁ mouse was found to be 45 mg/kg (Experiment 1).

Dose-dependent suppression of all haemopoietic cell lines was demonstrated following a single BU dose. The suppression and rebound of erythropoiesis and myelopoiesis was studied over a 10 d period and the relationship of HFR and LUC to erythropoietic and myelopoietic recovery were described (Experiment 3). The MTD for repeat BU dosing at fortnightly intervals was found to be 40 mg/kg (Experiment 2). At this dose level, a mild macrocytic anaemia and cytopenias of other lineages were seen 42 d after the dosing period.

The MTD of CAPS in the drinking water was found to be 4.0 mg/mL in the B6C3F₁ mouse (Experiment 4). At this dose level, haemoconcentration was present after five d dosing, but with resolution after 20 d continued dosing (Experiment 5). No haemotoxicity was evident at this dose level.

In the consecutive treatment of B6C3F₁ mice with BU and CAPS (Experiment 6), long-term BU-induced haemotoxicity was characterised by persistent peripheral cytopenias and reduced splenic cellularity. Early deaths due to BU dosing were from a failure of haemopoiesis, whilst the subsequent development of lymphomas was responsible for late

deaths. The administration of CAPS in the drinking water at 4.0 mg/mL did not cause additional haemotoxicity.

Investigations into the kinetics of CAPS in the mouse demonstrated a diurnal pattern of water intake with low levels of CAP in the serum at mid-morning (Experiment 7).

Gavage dosing with CAPS showed rapid and complete conversion of CAPS to CAP (Experiment 8). With the iv administration of high-dose CAPS it was shown that very little CAP entered the marrow. Parallel investigations with orally-administered high-dose CAPS demonstrated apoptosis of myeloid and erythroid precursors and *ex-vivo* inhibition of CFU-E growth.

The haemotoxicity of orally administered high-dose CAPS was characterised in the B6C3F₁ mouse (Experiment 10). BU-treated mice were then given orally administered high-dose CAPS, but, as in Experiment 6, no additional haemotoxicity of CAPS could be demonstrated (Experiment 11).

9.1.2 Considerations Related to the Validation of a Model of Chloramphenicol-induced Aplastic Anaemia in the B6C3F₁ Mouse

In attempting to generate a model of human AA in B6C3F₁ mice with BU, it was not possible to demonstrate additional toxicity with the administration of CAPS, either in drinking water (Experiment 6), or when the antibiotic was given by gavage at a much greater concentration (Experiment 11).

These findings are in keeping with the report of Pazdernik and Corbett (1980) who were unable to demonstrate CAP-mediated aplasia in the first 3 months post BU dosing using BDF₁ mice. Similarly, Robin *et al.* (Robin *et al.*, 1981; Bhoopalam *et al.*, 1986) found

that CAP did not promote aplasia in the BU-treated CAF₁ mouse, although evidence was reported of an increase in the frequency of BU-induced lymphomas. (Lymphomas induced by BU were first demonstrated by Upton *et al.* (1961) in RF/Up mice). Robin *et al.* (1981) in their studies with BU and CAP were unable to demonstrate any alteration in peripheral counts in CAP-treated mice.

Nevertheless, the BU model of chronic hypoplastic marrow failure of Morley *et al.*, although being unsuccessful in the present studies, has been validated in the prediction of captopril agranulocytosis in man (Boyd *et al.*, 1982). Preclinical studies with captopril had demonstrated that mice and rats tolerated the drug at up to 100 times the human dose, and that there was no *in vitro* inhibitory effect on canine CFU-GM (Boyd *et al.*, 1982). Neither captopril, nor CAP, inhibited the growth of murine CFU-GM from untreated mice, nor did either drug have any inhibitory effect on CFU-GM from BU-treated mice (Boyd *et al.*, 1984). Recently, however, it has been shown that captopril reduces non-adherent CFU-GM and high-proliferative potential colony-forming cells, but not adherent cells, in long-term murine marrow cultures (Chisi *et al.*, 1999). This finding appears, at least in the case of captopril, to validate the use of the BU model; however, there seem to be no reports of the effects of CAP in either human or murine long-term marrow culture.

However, another aspect which has a bearing on these discussions is that it is possible that the case for CAP to cause AA in man has been overstated. Recent surveys of Western populations do not implicate CAP as a causative agent in AA (Young and Alter, 1994; Kaufmann *et al.*, 1996), but this may be due to the restricted indications for its use (Mary *et al.*, 1996). Nevertheless, in Hong Kong, where sales of CAP are high, AA is not associated with CAP (Kumana *et al.*, 1987). Early publications in the 1950s and 1960s

linking AA to CAP were largely composed of individual case reports, and, at that time, criteria for the diagnosis of AA were less stringent than they are today: it is now usual for risk-benefit surveys to be based on large samples with matched controls (case-control population studies). Indeed, this has lead some workers to the conclusion that CAP may not be responsible for AA at all (Schrezenmeier, 2000).

9.2 Potential Future Investigations: Busulphan- and Chloramphenicol-induced Haemopoietic Injury in the Mouse: Models for Human Disease

Up to 3 months after dosing with BU, some mice treated with BU became severely aplastic. By extrapolation from the criteria for human SAA (Camitta *et al.*, 1976, 1982b) the condition of these animals can be considered to be SAA.

In surviving BU-treated animals, residual haemopoietic injury due to BU was clearly present as assessed by the presence of mild but persistent cytopenias. These animals were clinically well, and outwardly appeared normal, except for the presence of fur greying (Chapter 5). Patients diagnosed with AA and SAA may be considered cured when their peripheral cell counts fall within the normal range for their age, and when these counts have been maintained for one month (Camitta, 2000). In the phase of chronic hypoplastic marrow failure, BU-treated mice therefore resemble “cured” human AA patients. Recent investigations with BU in the BALB/c mouse, which follow from the earlier studies reported in the present work, indicate that by manipulation of the dosing regime, a model of myelosuppression can be reliably achieved (Macharia *et al.*, 1999a, 1999b; Diamanti *et al.*, 1999). Consistent reductions in marrow cellularity and colony-forming activity are seen, and the occurrence of lymphoma, compared with the B6C3F₁ mouse, is relatively rare (Turton, personal communication).

In BU-treated mice, after the initial phase of aplasia during the three months following BU dosing, the development of lymphoma supervenes as the cause of ICD occurrence. As discussed in Chapter 5, this may be as a result of the growth advantage of abnormal clones of stem cells, analogous to the development of PNH in human AA.

Thus, in addition to the early myelosuppression followed by a rebound to near-normal values (Chapter 3), there are three distinct outcomes of BU treatment in B6C3F₁ mice. That is, firstly, aplasia resembling SAA in man, secondly, a chronic phase characterised by peripheral cytopenias, analogous to “cured” AA in man, and thirdly, lymphoma, corresponding to PNH, or perhaps MDS or acute leukaemia. Each of these outcomes, or stages, in BU-induced haemotoxicity in the mouse has potential for further characterisation, and development as models of human disease.

The spectrum of haematological changes induced by BU in the B6C3F₁ mouse is similar to that reported for benzene and 1,3-butadiene (Green *et al.*, 1981; Irons *et al.*, 1986; Irons *et al.*, 1993; USNTP, 1993; Farris *et al.*, 1997). Benzene and 1,3-butadiene are both associated with AA, MDS, acute leukaemia and lymphoma in man (Goldstein, 1977; Melnick and Huff, 1992; Snyder *et al.*, 1993) and with clastogenic effects in animals (Irons *et al.*, 1987; Snyder and Kalf, 1994). Changes to haemopoietic cell populations induced by both chemicals have been systematically investigated, in particular in relation to mechanisms of leukaemogenesis (Irons *et al.*, 1989; Irons and Stillman, 1993; Schattenberg *et al.*, 1994; Irons and Stillman, 1996), nevertheless, the need for an animal model of chemically- or drug-induced MDS/AML still exists (Snyder and Kalf, 1994).

Similarly, as detailed in Chapter 1, the status of cellular markers and cytokines is well-established in human AA, as are the proliferative properties of peripheral and marrow cells in short and long-term culture. These properties, and details of cytogenetic changes and potential late clonal events, could be established and characterised in the BU mouse model.

Drug-associated AA, which is amenable to immunosuppression, characterised by delayed onset and which can spontaneously resolve, may have an immune component (Section 1.1.5.4). Most drugs associated with AA are also more commonly implicated in agranulocytosis, for which there is overwhelming evidence for the involvement of immune processes (Utrecht, 1992; Young, 1996). However, whereas in the case of agranulocytosis, where specific drug-related antibodies have been demonstrated, there is little direct proof of immune mediation in AA. Nevertheless, the presence of increased numbers of activated T cells in AA patients (Zoumbos *et al.*, 1985) together with elevated concentrations of IFN γ and TNF α (Hinterberger *et al.*, 1988) and increased apoptosis of CD34 $^+$ cells (Philpott *et al.*, 1995; Callera and Falcão, 1997) are consistent with an immune pathophysiology for AA. Recently, increased apoptosis of c-kit $^+$ cells has been demonstrated in mice with BU-induced chronic bone marrow aplasia (Gibson *et al.*, 2000). Further characterisation of the murine BU model of marrow hypoplasia, by the demonstration of inhibitory cytokines, upregulation of Fas antigen and T cell activation status, would determine the relevance of BU-induced murine marrow hypoplasia to immune mediated AA in man. Furthermore, studies on the response of BU-induced chronically hypoplastic mice to later challenge with BU, or their response to immunosuppressive agents would be of interest.

Sanguineti *et al.* (1983) observed an increased frequency of lymphomas in BALB/c and C57BL/6N mice given CAP alone in drinking water. Lymphoma development was only evident after two years' dosing with CAP. These mouse strains are not associated with as high a rate of spontaneous lymphomas as the B6C3F₁ mouse (Frith *et al.*, 1983; Lang, 1989; Sheldon *et al.*, 1995). This would suggest that carcinogenicity studies with BU and high dose CAPS in these three mouse strains, or in mice deficient in the tumour-suppressing gene p53 (Donehower *et al.*, 1992), may be of value in the characterisation of murine models of lymphoma.

It has been shown that, in the duck, guinea-pig and mouse, the administration of CAP or CAPS causes cessation of erythropoiesis which may spontaneously resume in spite of continued CAP/CAPS dosing (Rigdon *et al.*, 1954; Havard *et al.*, 1999b; Turton *et al.*, 2000). In the present work, the erythropoietic rebound seen in Experiment 10 appeared to be mediated, at least in part, by the spleen (Chapter 7). In contrast to mice, rats sampled after 19 d oral dosing with high dose CAPS showed no reduction in reticulocyte count (Turton *et al.*, 1999). However at the highest dose level, 4000 mg/kg, haemoglobin was reduced and the reticulocyte count was increased. Although this increase in reticulocyte count was not statistically significant, this pattern of data suggests a recovery from transient erythroid toxicity. A frequently-observed phenomenon in preclinical repeat-dose studies in rats, with a variety of classes of drug, is an initial sharp reduction in reticulocyte count, which occurs in the first few days after the first dose (Andrews, personal observations). This is followed, with continued dosing, by a rebound reticulocytosis which is accompanied by increased splenic erythropoiesis. The significance of such transient erythroid toxicity is unclear. Presumably, a single cohort of erythroid progenitors is affected, but the exact mechanism is unknown. Whether such

toxicity has implications for the administration of these drugs in man is therefore uncertain, and deserves further investigation.

9.3 Future Strategies for the Investigation of Idiosyncratic Drug-induced Aplastic Anaemia in Man

9.3.1 Metabolic Predisposition to Drug-induced Aplastic Anaemia

Yunis and Bloomberg (1964) speculated on the likely underlying cause of CAP-induced AA in man, giving four possible mechanisms: (a) the inability to excrete the drug normally, leading to increased drug levels; (b) the selective concentration of the drug in bone marrow; (c) abnormal metabolism of the drug giving rise to toxic metabolites and (d) an inborn error of metabolism such as an enzyme deficiency causing susceptibility to the parent drug or its normal metabolites. The present work has shown that CAP is cleared rapidly, and is not found at high concentrations in the bone marrow in the B6C3F₁ mouse. However, future investigations will undoubtedly focus on the role of abnormal metabolism in idiosyncratic haemotoxicity.

Peripheral blood and bone marrow cells contain a number of enzyme systems thought to be involved in the generation of reactive, and therefore short-lived metabolites (Uetrecht, 1992). In addition to P450 enzymes which are found in low concentrations in lymphocytes and monocytes, and bone marrow (Hodges *et al.*, 2000), prostaglandin synthase is found in peripheral mononuclear cells (Uetrecht, 1992), and myeloperoxidase (MPO) is present in granulocyte precursors and lineage-negative CD34⁺ cells (Schattenberg *et al.*, 1994). Benzene appears to exert its haemotoxicity, directly or indirectly, via hydroquinone, produced in the marrow by peroxidation by MPO (Irons *et al.*, 1992; Schattenberg *et al.*, 1994; Medinsky *et al.*, 1996). MPO is also thought to be

involved in the generation of reactive metabolites from a number of compounds which may cause agranulocytosis and AA, including phenytoin, carbamazepine and phenylbutzone. CAP itself may be metabolised to a short-lived chloramine derivative by this system (Utrecht, 1992). The oxidation profile of various drugs has been reported to correlate well with their potential to cause agranulocytosis and AA (Liégeois *et al.*, 1999). On the other hand, 1,3- butadiene, which causes leukaemias and lymphomas in man and multi-organ carcinogenicity in mice, notably thymic lymphomas, does so via the reactive metabolite 3,4-epoxybutene, formed as an intermediate by liver microsomal cytochrome P450 monooxygenase (Melnick and Huff, 1992; Irons *et al.*, 1993).

Poor metabolisers and those whose capacity to excrete metabolites is compromised, for example, patients with renal failure, are at particular risk of concentration-related adverse events. Thus, many patients who developed agranulocytosis after captopril administration had some degree of renal impairment (Boyd *et al.*, 1982). Felbamate is presumed to cause AA via atropaldehyde, a metabolite normally excreted as a glutathione conjugate: it is therefore supposed that susceptible individuals are glutathione deficient (Thompson *et al.*, 1996; 1997). The propositions that CAP-induced AA may be mediated by the metabolite CAP-NO (Miller *et al.*, 1978; Yunis *et al.*, 1980a; 1980b; 1987), or by the bacterial metabolite dehydro-CAP (Jimenez *et al.*, 1987), or by P450 dehalogenated metabolites capable of protein acylation (Krishna *et al.*, 1981), all presuppose inherent metabolic defects in affected patients. However, there is only very limited specific evidence of AA patients who have been shown to be unable to metabolise toxic metabolites (Young, 1996).

It is known that genetic variation contributes to observed differences in both pharmacokinetics and pharmacodynamics of drugs. For example, 0.3 % of western populations are homozygous for reduced levels of thiopurine methyltransferase (Spielberg, 1996). Such individuals are unable to metabolise azathioprine to 6-mercaptopurine, and instead generate 6-thioguanine nucleotides, which can lead to marrow suppression. Indeed, the observation of CAP-associated AA in twins (Best, 1967; Nagao and Mauer, 1969) and the association with certain HLA-DR subclasses with drug-associated AA (Nakao, 1997), indicate a genetically mediated susceptibility in some AA patients. Genetic variation in cytochrome P450 (CYP) isoenzymes is well recognised: polymorphism in CYP2C9, CYP2C19 and CYP2D6 can result in deficiency (poor or slow metabolisers) or enhancement of enzyme activity (extensive or ultra-rapid metabolisers; van der Weide and Steijns, 1999). However, analysis of certain P450 metaboliser genotypes implicated in remoxipride and benzene metabolism (CYP2D6 and CYP2E1) revealed no difference in distribution between AA patients and normal controls (Marsh *et al.*, 1997; 1999a).

In preclinical carcinogenicity testing it is desirable to select species with a similar metabolism to man for a particular drug (EMA, 1997). Where the particular idiosyncracy of metabolism in man is unknown (as with CAP-induced AA), there are difficulties in the selection of an appropriate species (Turton *et al.*, 1999). For example, whilst DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) is carcinogenic in mice, rats and hamsters, its parent compound DDT (dichlorodiphenyltrichloroethane) is carcinogenic in mice and rats but not hamsters (Rossi *et al.*, 1983). Similarly, 1,3-butadiene is clastogenic in many mouse *in vitro* and *in vivo* assays, but is generally negative in rat studies (Jacobson-Kram and Rosenthal, 1995). Furthermore, within a given species, the choice of strain may be

critical in the demonstration of a given toxic lesion (Miller *et al.*, 1978; Nebert, 1981; Scales and Andrews, 1991; de Haan *et al.*, 1997; Festing *et al.*, 2000).

The particular difficulties in identifying human metabolic pathways implicated in idiosyncratic toxic reactions such as AA have in the past led some to conclude that no animal model can meet these specifications (Haak, 1980); nevertheless, the study of altered gene expression in AA patients may lead to the understanding of the metabolic processes underlying idiosyncratic toxicity from specific drugs (Pennie, 2000). In addition, *in vitro* toxicity assays are being developed which utilise reporter genes whose activation predicts potential toxic responses (Nuwasir *et al.*, 1999). Such assays could potentially enable the selection of an appropriate knockout strain, such as a P450 deficient mouse, in which the administration of a novel compound would cause toxicity which might otherwise not be detectable.

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Proceedings of a National Meeting on Experimental and Clinical Oncology, p 44 abstract no. 50

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Original Article

Characterisation of Busulphan-Induced Myelotoxicity in B6C3F₁ Mice Using Flow Cytometry*

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Abstract. Three experiments were carried out to investigate the myelotoxicity of busulphan in female B6C3F₁ mice using the Technicon H*1 and the Sysmex R-1000 flow cytometers, instruments which produce a full blood count and a differential leucocyte count, and an automated reticulocyte count, respectively. In Experiment 1, a single dose of busulphan was administered at levels from 0 to 60 mg/kg and blood parameters measured at day 14. In Experiment 2, four doses of busulphan, from 0 to 40 mg/kg, were given at fortnightly intervals, and blood samples taken at days 14 and 42. In the third experiment, a single dose of busulphan was given at 0, 35 or 45 mg/kg and sequential blood, marrow and spleen samples examined up to day 10.

In the first experiment there was a dose-related depression in the numbers of all leucocyte types. Values for Hb, RBC and HCT were not affected, whereas MCV and percentage macrocytic erythrocytes were increased, and MCHC was decreased, at high dose levels. Platelet numbers showed marked dose-related decreases. There were dose-related decreases in the numbers of all leucocyte types in Experiment 2 at days 14 and 42. Large unstained cell (LUC) numbers were reduced, and the mean neutrophil peroxidase index (MPXI) was increased, at high busulphan levels. Hb, RBC and HCT were reduced, whereas MCV, MCH and percentage macrocytic and percentage hypochromic erythrocytes were increased, in a dose-related fashion. Reticulocyte numbers showed a dose-related upward trend, but platelet counts illustrated a dose-related

decrease, at days 14 and 42. In Experiment 3, busulphan caused a depression with a 'U'-shaped curve, in the numbers of monocytes, eosinophils, lymphocytes and neutrophils. Decreased values and 'U'-shaped curves were also seen for Hb, RBC, HCT and reticulocyte counts. Reticulocyte fluorescence ratio analysis showed that the high fluorescence ratio (HFR) was affected first and most profoundly. Calculation of the reticulocyte maturation index also demonstrated a dose-related effect on the earliest reticulocytes, and a 'rebound' effect. Total nucleated cell counts of the spleen and femur showed decreasing cell numbers and 'U'-shaped responses with 45 mg/kg busulphan.

This series of three experiments has established the use of a 6 week dosing regimen, with busulphan administered at fortnightly intervals, to induce myelotoxicity in a range of haematological parameters in female B6C3F₁ mice. We consider the use of the newly-developed flow cytometers and associated software, and the measurement of 'non-standard parameters' such as LUC, HFR and MPXI, to be particularly effective in the characterisation of these busulphan-induced haematological changes.

Keywords: Bone marrow; Busulphan; Cell counting; Haematopoiesis; Mouse; Toxicity

Introduction

In a series of experiments, Morley and his co-workers established a model of chronic hypoplastic bone marrow failure in BALB/c mice by the intraperitoneal administration of high levels of the cytotoxic drug busulphan

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(Morley and Blake 1974a,b; Morley et al. 1978). Several dosing protocols were investigated (Trainor and Morley 1976), but in one frequently-used regimen a total of 90 mg/kg of the agent was administered in four divided doses at two-weekly intervals. This six-week dosing procedure allowed the mice time for recovery from the acute effects of the drug after each administration (Morley and Blake 1974a). After the dosing period, the mice were left without further treatment for a six to eight week observation period. In the twelve months following busulphan administration, studies on haematological values revealed, to varying extents, depending on the particular parameter examined, a persistent effect on haematopoiesis. This effect on blood formation had hitherto not been fully appreciated as a sequel to the administration of anti-cancer drugs (Skipper et al. 1964).

Morley and Blake (1974a) reported that mortality in busulphan-treated mice was accelerated, in comparison with control animals. Death occurred at a variety of times after the dosing period, but it generally followed an acute illness and animals were found to have either profound cytopenias or lymphomas/leukaemias. Sequential blood sampling and haematocrit measurements in the post-dosing period demonstrated that where aplasia eventually occurred, anaemia had not persisted following busulphan treatment, but had developed with varying degrees of rapidity as a late stage event (Morley and Blake 1974a). In animals which had not yet succumbed to the terminal disease (mice were referred to as being in a 'latent' condition), peripheral blood changes were only slight. In these animals, any changes of haematocrit, and reticulocyte and platelet counts, were all within the control range, whereas the depression of leucocyte numbers was only slight to moderate. However, the cellularity of the bone marrow, and its capacity to form colonies either in *in vitro* systems or in sublethally irradiated recipient mice, were more markedly affected. Tibial counts in busulphan-treated 'latent' mice were approximately 58% of control values, CFC (Colony Forming Cells *in vitro*) were 42% and CFU-S (Colony Forming Unit-Spleen) were 25% of normal (Morley and Blake 1974b).

Since the time of Morley's reports, automated techniques for haematological examination have greatly improved in sensitivity of precision. Also, the volume of blood required to prime these automated instruments has decreased significantly. Two instruments in this category are the Technicon H*1 and the Sysmex R-1000. These are dedicated flow cytometers producing both a full blood count and a differential leucocyte count, and an automated reticulocyte count, respectively. Each of these instruments requires only 100 µl of whole blood for analysis. The Technicon H*1 has been in routine use in hospital laboratories since 1985, and recently software has been introduced specifically for several laboratory animal species (Davies and Fisher 1991). Similarly, a modified version of the clinically used Sysmex R-1000 is now available for reticulocyte analysis in non-human species (Fuchs and Eder 1991).

Therefore, using these machines, it is now possible to perform large numbers of haematological measurements routinely. Many of these measurements, for example the assessment of cell counts and some erythrocyte sizing and haemoglobin-associated indices, make up the standard haematological profile that has been carried out in toxicological laboratories for many years in the evaluation of laboratory animal blood samples. However, in addition to the standard haematological profile, these flow cytometers generate data automatically on a number of other measurements which have not been available previously (i.e. 'non-standard parameters').

For a series of experiments on drug-induced haematological changes, we wished to establish a dosing regimen with busulphan in mice, based on Morley's investigations (Morley and Blake 1974a,b; Morley et al. 1978). Experiments were to be carried out in female B6C3F₁ mice, and busulphan administered on four separate occasions over a six-week period. In view of the relatively minor haematological changes reported by Morley in 'latent' mice after treatment with busulphan (Morley and Blake 1974a), it was considered that the availability of the newly developed flow cytometers and associated software represented an opportunity to investigate whether the blood profile of mice in the post-dosing period could be better characterised. In addition, it would also be possible to examine and evaluate the value of 'non-standard parameters', and whether there was any advantage in reporting them routinely in toxicological investigations.

Materials and Methods

Animals

Female B6C3F₁ mice (Glaxo Group Research Ltd), 12–14 weeks old, were housed in groups of 5 or 6, bedded on wood shavings, and given diet (Rat and Mouse No. 1, SDS Ltd, Witham, Essex) and drinking water *ad libitum*. A temperature of 22°C ± 1°C was maintained, with a relative humidity of 45%–70% and a 12:12 h light: dark cycle (lights on at 06.00 h). Animals were acclimatised for at least 5 days before each experiment and were observed daily, but more frequently immediately after dosing.

Busulphan Dosing

Busulphan (Sigma Chemical Co. Ltd, Poole, Dorset) was dissolved in acetone at a concentration of 16.7 mg/ml. The busulphan solution was administered by intraperitoneal injection after dilution with distilled water to give a constant dose volume of 10 ml/kg. Control mice were given acetone with distilled water at a dose volume of 10 ml/kg.

Trial Designs

Experiment 1: Single Dose Study. Single doses of busulphan were administered at 0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 mg/kg to four mice at each dose level. At 14 days, animals were killed by CO₂ overdose and 0.5 ml blood was removed from the posterior vena cava and anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacristan, Durham).

Experiment 2: Repeat Dose Study. Groups of 12 mice were given four doses of busulphan at fortnightly intervals at 0, 10, 15, 20, 25, 30, 35, and 40 mg/kg. Six mice in each group were killed by CO₂ overdose at 14 days after the last dose of the drug, and the remaining animals killed at 42 days after the last dose; blood was sampled as in Experiment 1.

Experiment 3: Single Dose Sequential Sampling Study. Thirty mice were each given a single dose of busulphan at 0, 35 or 45 mg/kg. At 1, 2, 3, 4, 7 and 10 days after dosing, five mice were killed from each of the three dosed groups and sampled as in Experiment 1. At post mortem, the contents of the right femur were flushed into isotonic saline, and a smear was prepared from the contents of the left femur. Spleens were removed, bisected and a contact preparation made on a glass slide with the cut face of the organ. The spleens were then scissor-minced and dispersed in isotonic saline by cavitation in a 50 ml syringe. A further five untreated mice were killed and sampled at the time of busulphan dosing ('day 0 controls').

Haematological Measurements

Blood samples, and bone marrow and spleen suspensions, were analysed using a Technicon H*1 haematology analyser (Bayer UK Ltd, Basingstoke, Hants) with mouse-specific software (Technicon, Swords, Co. Dublin, Eire). Measurements performed were: haemoglobin concentration (Hb), erythrocyte count (RBC), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), haemoglobin distribution width (HDW), percentage hypochromic erythrocytes (haemoglobin concentration <25 g/dl), percentage macrocytes (cell volume >70 fl), leucocyte count (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, large unstained cells (LUC), mean neutrophil peroxidase index (MPXI), platelet count, mean platelet volume (MPV), platelet distribution width (PDW). The H*1 is able to measure the separation of modal values of nuclear size between mononuclear and polymorphonuclear cells (the lobularity index). However, with rodent blood, the instrument is unable to discriminate separate populations owing to the preponderance of lymphocytes (mononuclear cells) in most samples. The lobularity index is therefore not reported in these studies.

For the marrow and spleen samples, total nucleated cell counts were obtained from the basophil channel of the H*1. WBC correlation errors were obtained due to the apparent low peroxidase channel count, which in turn is due to the relatively small size of some spleen and marrow cells, and the low peroxidase activity associated with immature cells. In contrast, the basophil channel count is due entirely to stripped nuclei.

Reticulocyte analysis was performed using a Sysmex R-1000 (Toa Medical Electronics Ltd, Milton Keynes, Bucks). The output voltage gain setting (using a GAU-1 module, Toa Medical Electronics Ltd) was as for the analysis of human samples, as determined by local evaluation (unpublished results). Absolute numbers of reticulocytes were obtained by combining the RBC count obtained using the H*1 with the percentage reticulocyte count from the R-1000. Three equal divisions of the total number of reticulocytes gave the (percentage) low (L), medium (M) and high fluorescence ratio (HFR). The reticulocyte maturation index (RMI) was calculated as proposed by Tatsumi and Tsuda (1991): RMI = [(MFR + HFR)/LFR] × 100%.

Marrow smears and spleen impressions were stained with May-Grunwald-Geimsa stain, and differential counts performed by eye on 200 cells.

Statistical Analysis

Student's *t* test was used to compare treated and control groups.

Results

Experiment 1: Single Dose Study

The majority of animals dosed with 50, 55 or 60 mg/kg busulphan died, or were killed *in extremis*, in the 24 h following drug administration, and the remainder in the 24–96 h period after dosing. Haematological data from these mice are not presented. Animals dosed at 40 and 45 mg/kg showed transient effects of toxicity.

For animals given 10–45 mg/kg busulphan, the haematology at 14 days after dosing is presented in Table 1. There was a dose-related depression in the numbers of most leucocyte types. The reduction in neutrophil counts with increasing busulphan dose levels was accompanied by an upward trend in the MPXI. At 25 mg/kg busulphan and above, there was a decrease in monocyte numbers, but an accompanying decrease in the LUC count only reached statistical significance at 35 mg/kg. There was an increase in lymphocyte numbers in the range of 10–25 mg/kg busulphan.

Except for some minor effects, busulphan administration at 10–40 mg/kg did not affect the values of Hb, RBC or HCT. At 45 mg/kg, one mouse in the group of four animals had markedly reduced values for each of these three parameters, whereas the values for the other three individuals were normal. However, results for MCV

Table 1. Haematological results^a from 12- to 14-week-old female B6C3F₁ mice dosed intraperitoneally with 10–45 mg/kg busulphan and killed 14 days later^b

Control (vehicle)	Dose levels of busulphan (mg/kg)							
	10	15	20	25	30	35	40	45
Hb (g/dl)	15.7 (0.3)	15.6 (0.2)	15.6 (0.5)	16.2 (0.5)	15.7 (0.5)	15.2 (0.3)*	15.7 (0.2)	15.5 (0.6)
RBC ($\times 10^{12}/l$)	9.79 (0.19)	9.71 (0.19)	9.78 (0.30)	10.22 (0.35)*	9.92 (0.28)	9.52 (0.19)	9.77 (0.12)	9.80 (0.35)
HCT (l/l)	0.540 (0.013)	0.520 (0.010)	0.525 (0.018)	0.543 (0.019)	0.532 (0.020)	0.530 (0.006)	0.543 (0.004)	0.547 (0.022)
MCV (fl)	55.2 (0.3)	53.5 (0.6)**	53.7 (0.8)**	53.1 (0.3)***	53.7 (0.8)**	55.8 (0.8)	55.6 (0.8)	55.8 (0.4)*
MCH (pg)	16.0 (0.1)	16.1 (0.1)	15.9 (0.1)	15.9 (0.1)	15.9 (0.2)	16.0 (0.2)	16.1 (0.2)	15.8 (0.1)**
MCHC (g/dl)	29.1 (0.3)	30.0 (0.2)***	29.7 (0.2)**	29.9 (0.2)***	29.6 (0.3)*	28.6 (0.6)	28.9 (0.1)	28.3 (0.2)***
RDW (%)	11.8 (0.4)	12.4 (1.1)	11.7 (0.2)	12.5 (1.2)	11.4 (0.2)	12.1 (0.8)	11.8 (0.2)	12.9 (0.7)*
HDW (g/dl)	1.60 (0.04)	1.58 (0.13)	1.56 (0.02)	1.59 (0.09)	1.52 (0.02)*	1.64 (0.14)	1.48 (0.03)**	1.51 (0.02)**
Macro (%)	0.1 (0.0)	0.2 (0.1)*	0.3 (0.1)**	0.2 (0.0)**	0.2 (0.1)*	0.9 (0.5)*	0.9 (0.4)**	1.1 (0.4)***
Hypo (%)	0.2 (0.1)	3.3 (1.6)**	3.2 (1.9)*	2.5 (1.0)***	2.0 (0.7)***	11.0 (6.1)	5.0 (1.4)***	7.7 (3.0)***
Retic (% red blood cells)	2.79 (1.22)	2.25 (0.23)	2.99 (0.45)	2.18 (0.40)	2.50 (0.24)	3.28 (0.54)	3.05 (0.10)	3.18 (0.41)
Retic ($\times 10^9/l$)	275.5 (122.1)	218.0 (27.0)	296.0 (44.0)	223.0 (49.0)	248.0 (23.3)	312.1 (49.1)	297.6 (11.5)	311.8 (39.3)
LFR (%)	53.7 (4.4)	55.5 (8.9)	47.9 (5.5)	56.6 (7.2)	49.4 (4.9)	54.7 (5.7)	55.4 (5.2)	57.3 (2.6)
MFR (%)	32.4 (1.8)	31.5 (5.8)	36.9 (3.9)*	32.6 (3.2)	35.6 (2.3)*	31.5 (1.5)	32.1 (2.0)	32.4 (1.2)
HFR (%)	13.7 (3.6)	13.0 (3.3)	15.1 (4.8)	10.8 (4.8)	15.0 (3.5)	13.8 (4.4)	12.4 (3.6)	10.4 (2.1)
WBC ($\times 10^9/l$)	6.8 (1.1)	10.0 (1.2)**	9.9 (0.5)***	10.7 (2.0)**	9.2 (1.9)*	7.9 (2.2)	5.5 (0.9)	6.7 (1.7)
Neut ($\times 10^9/l$)	0.64 (0.15)	0.73 (0.08)	0.85 (0.07)*	0.75 (0.23)	0.70 (0.20)	0.77 (0.31)	0.49 (0.12)	0.36 (0.04)**
Lymph ($\times 10^9/l$)	5.43 (0.89)	8.52 (1.04)***	8.38 (0.55)***	9.10 (1.72)**	7.96 (1.62)*	6.62 (1.58)	4.68 (0.77)	5.93 (1.61)
Mono ($\times 10^9/l$)	0.22 (0.05)	0.26 (0.07)	0.23 (0.04)	0.26 (0.03)	0.18 (0.04)	0.14 (0.10)	0.10 (0.02)**	0.12 (0.05)*
Eos ($\times 10^9/l$)	0.27 (0.07)	0.16 (0.05)*	0.11 (0.06)*	0.23 (0.11)	0.15 (0.07)*	0.11 (0.09)*	0.10 (0.04)**	0.12 (0.05)*
Baso ($\times 10^9/l$)	0.04 (0.02)	0.06 (0.02)	0.05 (0.01)	0.05 (0.02)	0.04 (0.01)	0.02 (0.02)	0.01 (0.01)*	0.03 (0.02)
LUC ($\times 10^9/l$)	0.20 (0.03)	0.24 (0.08)	0.22 (0.07)	0.24 (0.03)	0.19 (0.06)	0.20 (0.15)	0.13 (0.04)**	0.16 (0.05)
MPXI	-17.8 (1.6)	-17.6 (2.3)	-18.7 (0.9)	-18.3 (1.7)	-19.0 (0.9)	-16.8 (1.9)	-17.2 (0.3)	-17.7 (0.9)
PLTS ($\times 10^9/l$)	1191.5 (49.3)	944.3 (74.9)***	858.5 (20.4)***	807.8 (244.8)**	775.3 (84.1)***	707.5 (98.0)***	544.0 (37.9)***	537.2 (83.9)***
MPV (fl)	4.2 (0.1)	4.6 (0.2)**	4.6 (0.2)*	4.4 (0.1)	4.5 (0.3)	4.6 (0.1)*	4.5 (0.1)*	4.7 (0.4)*
PDW (%)	55.9 (1.1)	51.4 (1.6)***	51.4 (2.3)**	53.2 (0.9)**	52.9 (1.9)*	53.7 (1.7)*	54.8 (1.3)	54.2 (4.3)

^a Values are means, SD in parentheses; $n=4$ at each busulphan dose level.^b Abbreviations: Hb, haemoglobin; RBC, red blood cells; HCT, haematocrit; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width; HDW, haemoglobin distribution width; Macro, macrocytic erythrocytes; Hypo, hypochromic erythrocytes; Retic, reticulocytes; LFR, low fluorescence ratio; MFR, medium fluorescence ratio; HFR, high fluorescence ratio; WBC, white blood cells; Neut, neutrophils; Lymph, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; LUC, large unstained, cells; MPXI, mean neutrophil peroxidase index; PLTS, platelets; MPV, mean platelet volume; PDW, platelet distribution width.* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

showed an increase at 30 mg/kg busulphan and above, whereas at 10–25 mg/kg the values were significantly lower. The findings for MCHC demonstrated a significant decrease at 40 and 45 mg/kg but there was an increase in the values at 10–25 mg/kg of the drug. The percentage of hypochromic erythrocytes and macrocytic erythrocytes were increased at all busulphan dose levels. The value for MCH was significantly decreased at 40 mg/kg busulphan, but there was no clear effect at lower dose levels of the drug. RDW was not affected by busulphan treatment, except for a significantly increased value at the 45 mg/kg dose level. At 25, 35 and 40 mg/kg busulphan, values for HDW were reduced.

Reticulocyte numbers showed an upward trend with increasing busulphan dosage, but apart from increases in MFR at 15 and 25 mg/kg, statistical analysis of the percentage LFR, MFR and HFR revealed no busulphan-induced effects (Table 1). Platelet numbers showed a marked decrease with increasing busulphan dose levels. Values for MPV were slightly increased, and for PDW slightly decreased, at some busulphan levels, but the effects were not dose-dependent. At 45 mg/kg, three individuals had normal values for MPV and PDW, however the fourth, an anaemic mouse (mentioned above) had a reduced value for MPV and an increase in PDW.

Experiment 2: Repeat Dose Study

Busulphan was administered in four fortnightly occasions at levels from 10 to 40 mg/kg and Tables 2 and 3 give haematological results at 14 and 42 days after the final dose of drug, respectively. In general, the administration of busulphan caused a dose-related decrease in the numbers of all types of leucocyte at days 14 and 42; the effects were particularly evident at 30, 35 and 40 mg/kg busulphan. As in Experiment 1, there was a tendency for lymphocyte numbers to be higher at low busulphan dose levels; this was particularly evident at 15 mg/kg at both day 14 and day 42. However, the monocyte counts at day 14 showed clear dose-related reductions at 25 mg/kg busulphan and above (Table 2), but at day 42 the cell count was only reduced to a statistically significant level at 40 mg/kg (Table 3). There was a tendency for a reduction in the LUC count, at 14 and 42 days, at 30 mg/kg busulphan and above. However, the MPXI showed increasing values at 30, 35 and 40 mg/kg busulphan, at both time points.

At both day 14 and day 42 there were dose-related reductions in Hb, RBC and HCT values, especially at 30 mg/kg busulphan and above. However, there was also a slight increase in Hb, RBC and HCT measurements at day 42, notably at 15 mg/kg busulphan (Table 3). Values for MCV, MCH, percentage macrocytic erythrocytes and percentage hypochromic erythrocytes increased in a dose-related fashion as the busulphan level was raised. There were no busulphan-induced effect on RDW or on HDW at 14 or 42 days, apart from a significant decrease in RDW at 35 mg/kg at day 42. Reticulocyte numbers

showed a dose-related upward trend at day 14 and day 42 after the final administration of busulphan, and at both times there appeared to be a tendency for the higher reticulocyte numbers to be due to an increase in the percentage HFR and a concomitant decrease in the percentage LFR. However, only at 42 days did some changes in the fluorescence fractions achieve statistical significance (Table 3). Platelet numbers decreased significantly in a dose-related fashion at 10 mg/kg busulphan and above, at 14 and at 42 days. In treated mice at day 14, MPV was unaffected, but at day 42, values were decreased, but not significantly, in animals given 35 and 40 mg/kg busulphan. In mice dosed with 25 mg/kg busulphan and above, PDW was significantly increased at day 14 after dosing (Table 2). At day 42, PDW was increased in mice receiving 35 and 40 mg/kg busulphan, but the increase did not reach statistical significance (Table 3).

Experiment 3: Single Dose Sequential Sampling Study

Results for several haematological parameters are presented in Figs. 1–9. The administration of a single dose of busulphan at 35 or 45 mg/kg caused a depression in the counts of several leucocyte types. For the monocyte count (Fig. 1), the reduction in cell count was pronounced on day 2, but for the eosinophil and lymphocyte counts, decreases occurred slightly later, on day 3. The reductions in the monocyte and eosinophil counts were dose related. However, a dose-related effect was not so clear in the case of the lymphocyte counts. The monocyte and eosinophil counts remained at significantly lowered levels until day 10, but this response was not so clearly evident for the other white blood cell types. In the neutrophil count there was only a conspicuous decrease at 45 mg/kg busulphan, which achieved statistical significance at day 4. The MPXI was unaffected in mice treated with 35 mg/kg busulphan, but at 45 mg/kg the index was increased at day 7. Basophil counts showed no treatment-related changes. In general terms, the effect on all white cell types was to produce a 'U'-shaped curve, with partial or complete recovery by day 10.

Values for Hb, RBC, HCT also showed dose-related 'U'-shaped responses, but by day 10 the values for these three parameters had not returned to those of the control mice (for RBC see Fig. 2). Busulphan treatment did not affect values for MCV, but increased numbers of macrocytes were evident at days 7 and 10. No treatment-related changes were evident in values for RDW, HDW, MCH, MCHC or percentage hypochromic erythrocytes.

The reticulocyte counts produced marked 'U'-shaped responses, at both 35 and 45 mg/kg busulphan (Fig. 3). At each dose level the decreases were maximal at day 3 post-dosing, and at 45 mg/kg the counts were reduced almost to zero at this time. These counts, at 35 and 45 mg/kg busulphan, rebounded to levels above the baseline values at day 7, and then fell slightly to day 10. The

Table 2. Haematological results from 12- to 14-week-old female B6C3F₁ mice dosed intraperitoneally with busulphan on four fortnightly occasions at 10–40 mg/kg and killed at 14 days after the final dose of drug^a

	Dose levels of busulphan (mg/kg)							
	Control (vehicle)	10	15	20	25	30	35	40
Hb (g/dl)	15.2 (0.3)	15.3 (0.4)	15.2 (0.2)	15.2 (0.3)	15.2 (0.6)	14.6 (0.6)	14.2 (0.4)*	13.2 (1.5)*
RBC ($\times 10^{12}/l$)	9.99 (0.23)	9.77 (0.28)	9.71 (0.22)	9.62 (0.21)	9.59 (0.40)	9.07 (0.27)***	8.72 (0.31)***	7.92 (0.94)**
HCT (l/l)	0.509 (0.120)	0.505 (0.016)	0.503 (0.008)	0.508 (0.016)	0.510 (0.019)	0.491 (0.015)	0.483 (0.015)*	0.446 (0.048)*
MCV (fl)	51.0 (0.1)	51.6 (0.1)**	51.9 (0.6)*	52.8 (1.0)*	53.2 (0.4)***	54.2 (0.4)***	55.4 (0.5)***	56.4 (0.9)***
MCH (pg)	15.2 (0.3)	15.6 (0.0)	15.6 (0.3)*	15.8 (0.1)***	15.9 (0.2)*	16.0 (0.3)**	16.3 (0.2)***	16.7 (0.3)***
MCHC (g/dl)	29.8 (0.5)	30.3 (0.1)	30.2 (0.4)	30.0 (0.5)	29.8 (0.3)	29.6 (0.4)	29.5 (0.3)	29.6 (0.4)
RDW (%)	14.9 (2.7)	11.5 (0.1)	13.1 (2.8)	13.7 (2.3)	11.6 (0.1)	12.6 (0.5)	12.9 (0.3)	14.9 (2.3)
HDW (g/dl)	1.65 (0.15)	1.51 (0.04)	1.60 (0.11)	1.66 (0.18)	1.55 (0.03)	1.61 (0.13)	1.58 (0.04)	1.70 (0.12)
Macro (%)	0.0 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)*	0.2 (0.1)*	0.6 (0.3)**	1.3 (0.5)**	4.3 (3.6)
Hypo (%)	0.8 (0.4)	0.6 (0.4)	1.0 (0.4)	1.8 (2.0)	3.0 (2.3)	3.2 (1.4)*	6.4 (4.0)*	4.2 (2.1)*
Retic (% red blood cells)	2.98 (0.87)	2.50 (0.64)	3.18 (0.74)	2.98 (0.65)	2.96 (0.49)	2.86 (0.05)	4.07 (0.86)	4.50 (1.18)
Retic ($\times 10^9/l$)	298.6 (92.9)	243.0 (56.0)	310.0 (74.0)	286.0 (61.0)	284.0 (46.0)	260.0 (44.0)	354.0 (72.0)	348.0 (62.0)
LFR (%)	53.8 (6.0)	57.1 (2.9)	50.4 (3.3)	51.6 (3.6)	54.2 (5.0)	54.6 (4.8)	50.6 (4.7)	48.6 (3.1)
MFR (%)	31.4 (2.6)	33.1 (0.1)	33.4 (1.7)	33.3 (2.6)	32.0 (2.7)	31.7 (1.6)	32.0 (3.3)	30.8 (3.0)
HFR (%)	14.9 (3.8)	9.9 (3.0)	16.2 (3.2)	15.1 (4.1)	13.8 (3.2)	13.8 (3.6)	17.5 (2.0)	20.6 (5.2)
WBC ($\times 10^9/l$)	8.7 (3.6)	9.0 (1.3)	10.1 (1.3)	8.8 (0.9)	8.4 (2.1)	6.7 (1.6)	5.5 (1.7)	4.9 (1.6)*
Neut ($\times 10^9/l$)	1.30 (1.10)	0.89 (0.01)	1.02 (0.19)	0.98 (0.15)	0.74 (0.23)	0.63 (0.39)	0.45 (0.13)	0.48 (0.41)
Lymph ($\times 10^9/l$)	6.44 (2.25)	7.44 (1.01)	8.27 (1.00)	7.08 (0.80)	7.05 (1.79)	5.66 (1.27)	4.63 (1.58)	4.14 (1.49)
Mono ($\times 10^9/l$)	0.38 (0.13)	0.25 (0.07)	0.30 (0.08)	0.27 (0.04)	0.22 (0.05)*	0.17 (0.05)*	0.12 (0.03)**	0.09 (0.04)***
Eos ($\times 10^9/l$)	0.22 (0.08)	0.16 (0.11)	0.20 (0.09)	0.20 (0.05)	0.12 (0.09)	0.10 (0.05)*	0.07 (0.04)**	0.08 (0.05)*
Baso ($\times 10^9/l$)	0.05 (0.05)	0.03 (0.01)	0.05 (0.00)	0.04 (0.02)	0.04 (0.01)	0.03 (0.00)	0.04 (0.02)	0.03 (0.01)
LUC ($\times 10^9/l$)	0.26 (0.23)	0.20 (0.06)	0.27 (0.08)	0.21 (0.04)	0.19 (0.05)	0.15 (0.03)	0.15 (0.09)	0.11 (0.04)
MPXI	-9.5 (1.7)	-8.3 (1.4)	-10.5 (1.6)	-11.1 (2.6)	-8.2 (3.9)	-3.0 (8.5)	-5.1 (3.9)	-1.9 (8.1)
PLTS ($\times 10^9/l$)	1061.8 (59.6)	795.0 (78.0)*	726.0 (107.0)**	800.0 (46.0)***	722.0 (98.0)***	495.0 (129.0)***	384.0 (69.0)***	176.0 (143.0)***
MPV (fl)	4.7 (0.2)	4.8 (0.2)	4.7 (0.1)	4.5 (0.2)	4.5 (0.2)	4.8 (0.3)	4.9 (0.3)	4.7 (0.5)
PDW (%)	51.3 (1.7)	51.6 (2.2)	51.8 (1.7)	53.8 (2.3)	54.9 (2.1)*	54.9 (2.1)*	55.8 (2.8)*	62.9 (8.8)*

^aFootnotes as Table 1, except $n=6$ at each busulphan dose level.

Table 3. Haematological results from 12- to 14-week-old female B6C3F₁ mice dosed intraperitoneally with busulphan on four fortnightly occasions at 10–40 mg/kg and killed at 42 days after the final dose of drug^a

Control (vehicle)	Dose levels of busulphan (mg/kg)							
	10	15	20	25	30	35	40	
Hb (g/dl)	14.9 (0.3)	15.6 (0.7)*	15.5 (0.4)*	15.2 (0.2)	15.0 (0.5)	14.7 (0.5)	14.8 (0.5)	13.7 (1.8)
RBC ($\times 10^{12}/l$)	9.68 (0.24)	10.06 (0.41)	10.11 (0.28)*	9.93 (0.08)	9.66 (0.36)	9.27 (0.24)*	9.22 (0.25)*	8.34 (1.30)*
HCT (l/l)	0.498 (0.012)	0.524 (0.029)	0.518 (0.013)*	0.512 (0.008)	0.499 (0.019)	0.494 (0.014)	0.495 (0.012)	0.465 (0.066)
MCV (fl)	51.4 (0.6)	52.0 (0.8)	51.2 (0.7)	51.5 (0.5)	51.7 (0.8)	53.3 (0.9)**	53.7 (0.7)***	55.8 (2.4)**
MCH (pg)	15.3 (0.1)	15.5 (0.3)	15.4 (0.1)	15.3 (0.2)	15.5 (0.3)	15.8 (0.4)*	16.1 (0.2)***	16.5 (0.5)***
MCHC (g/dl)	29.9 (0.3)	29.9 (0.4)	30.0 (0.3)	29.7 (0.5)	30.0 (0.2)	29.7 (0.6)	29.9 (0.3)	29.6 (0.8)
RDW (%)	11.7 (0.1)	12.1 (0.5)	12.1 (0.8)	12.4 (1.3)	12.5 (1.3)	12.0 (1.2)	11.4 (0.2)*	12.4 (1.0)
HDW (g/dl)	1.52 (0.02)	1.50 (0.04)	1.58 (0.09)	1.60 (0.15)	1.57 (0.16)	1.56 (0.17)	1.50 (0.04)	1.56 (0.05)
Macro (%)	0.1 (0.0)	0.1 (0.0)	0.1 (0.1)	0.1 (0.0)	0.1 (0.0)	0.2 (0.2)	0.1 (0.0)	1.7 (3.1)
Hypo (%)	1.7 (0.8)	2.2 (1.5)	1.5 (0.7)	2.2 (1.2)	1.8 (1.0)	3.3 (0.5)***	3.0 (1.4)	4.7 (2.1)*
Retic (% red blood cells)	2.33 (0.80)	2.75 (0.43)	2.74 (0.60)	3.14 (0.94)	3.00 (0.36)	3.20 (0.37)	2.85 (0.21)	3.43 (1.73)
Retic ($\times 10^9/l$)	226.0 (76.0)	229.0 (85.0)	277.0 (58.0)	315.0 (97.0)	292.0 (37.0)	301.0 (38.0)	252.0 (47.0)	282.0 (107.0)
LFR (%)	58.1 (1.8)	52.7 (3.7)*	57.2 (5.5)	55.6 (5.0)	56.9 (3.5)	51.6 (1.5)***	53.6 (3.3)*	48.6 (11.7)
MFR (%)	27.3 (2.0)	30.1 (2.1)*	28.2 (2.2)	29.7 (1.3)	27.7 (2.7)	29.1 (2.0)	28.8 (1.0)	28.3 (1.6)
HFR (%)	14.7 (3.0)	17.2 (3.5)	14.3 (4.0)	14.7 (4.1)	15.4 (1.1)	19.4 (3.2)*	17.6 (2.7)	18.1 (2.9)
WBC ($\times 10^9/l$)	8.2 (1.4)	8.0 (1.4)	8.4 (0.8)	7.8 (1.3)	7.7 (1.6)	5.7 (0.9)*	5.4 (1.3)**	3.8 (1.5)***
Neut ($\times 10^9/l$)	0.97 (0.37)	1.07 (0.24)	0.95 (0.20)	0.97 (0.36)	1.32 (0.67)	0.92 (0.47)	0.62 (0.11)	0.64 (0.31)
Lymph ($\times 10^9/l$)	6.46 (1.15)	6.47 (1.28)	6.76 (0.68)	6.14 (0.86)	5.70 (0.97)	4.22 (1.26)*	4.28 (1.14)*	2.73 (1.09)***
Mono ($\times 10^9/l$)	0.32 (0.08)	0.31 (0.04)	0.29 (0.05)	0.28 (0.03)	0.34 (0.09)	0.26 (0.03)	0.25 (0.05)	0.19 (0.10)*
Eos ($\times 10^9/l$)	0.14 (0.03)	0.16 (0.09)	0.20 (0.08)	0.17 (0.06)	0.17 (0.09)	0.18 (0.04)	0.17 (0.05)	0.14 (0.06)
Baso ($\times 10^9/l$)	0.03 (0.01)	0.04 (0.01)	0.03 (0.01)	0.03 (0.02)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)*	0.02 (0.01)*
LUC ($\times 10^9/l$)	0.14 (0.04)	0.15 (0.02)	0.13 (0.05)	0.16 (0.06)	0.14 (0.05)	0.09 (0.02)	0.08 (0.03)*	0.08 (0.02)*
MPXI	-5.1 (2.8)	-4.7 (4.3)	-6.5 (2.2)	-5.1 (6.0)	-7.4 (3.3)	-0.3 (6.9)	0.1 (8.6)	0.4 (9.3)
PLTS ($\times 10^9/l$)	1211.0 (135.0)	742.0 (177.0)***	731.0 (358.0)*	857.0 (94.0)**	843.0 (122.0)**	660.0 (190.0)***	654.0 (346.0)*	478.0 (375.0)**
MPV (fl)	4.6 (0.1)	4.8 (0.3)	4.6 (0.5)	4.6 (0.1)	4.6 (0.3)	4.6 (0.3)	4.3 (0.5)	4.2 (0.8)
PDW (%)	53.4 (1.2)	50.6 (2.9)*	53.5 (3.7)	52.9 (0.7)	52.7 (2.5)	52.3 (2.5)	55.8 (2.8)	62.9 (8.8)

^aFootnotes as Table 1, except $n=6$ at each busulphan dose level.

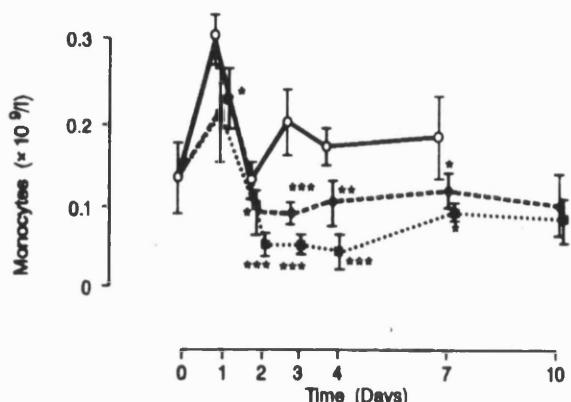


Fig. 1. Monocyte counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; ** p <0.01; *** p <0.001.

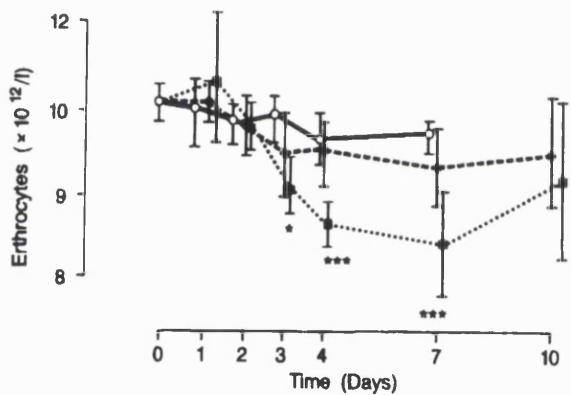


Fig. 2. Erythrocyte counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; *** p <0.001.

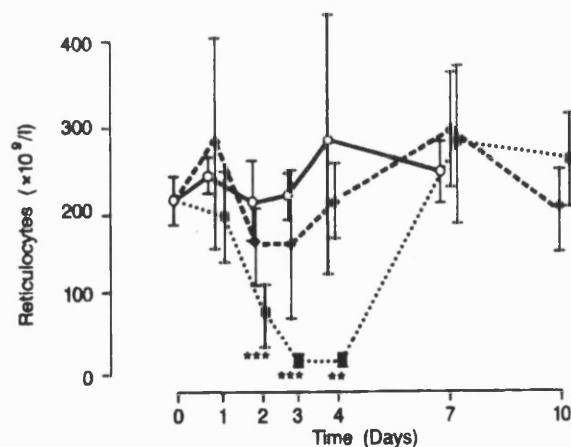


Fig. 3. Reticulocyte counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. ** p <0.01; *** p <0.001.

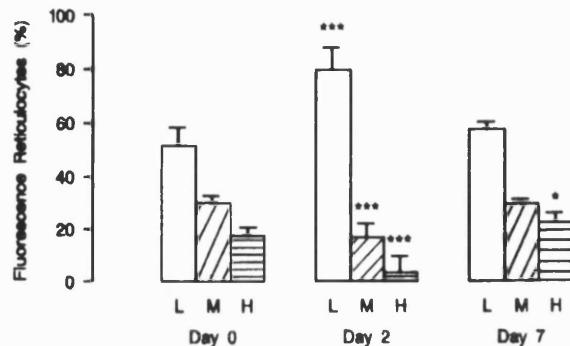


Fig. 4. Relative proportions (as %) of low (L), medium (M) and high (H) fluorescence ratio reticulocytes in female B6C3F₁ mice on day 0 (untreated control animals), and in mice given 45 mg/kg busulphan intraperitoneally and killed at two and seven days postdosing. Each bar represents the mean of values from five animals postdosing, \pm SD. * p <0.05; *** p <0.001.

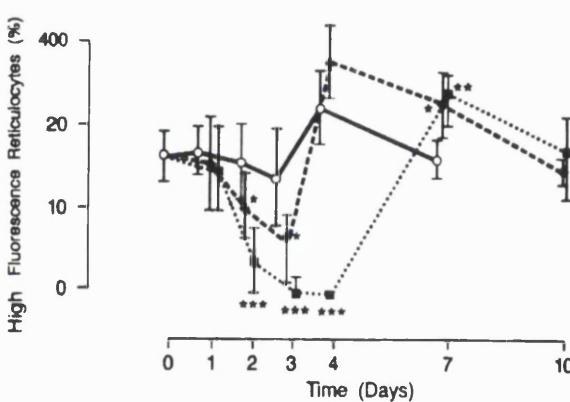


Fig. 5. High fluorescence ratio (HFR) reticulocyte counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; ** p <0.01; *** p <0.001.

analysis of reticulocyte fluorescence ratios (Fig. 4) showed that the percentage HFR (Fig. 5), i.e. the most immature cells, was affected first, and most profoundly, with very low levels being seen at day 2 at 45 mg/kg busulphan. Calculations of the RMI (Fig. 6) also illustrated very clearly the dose-related effect on the earliest reticulocytes and also the 'rebound' effect at both busulphan dose levels. The rebound phenomenon was much more obvious in the percentage HFR and RMI than in the total reticulocyte count. This was particularly true for the 35 mg/kg group. The data appeared somewhat less widely distributed in the percentage HFR analysis, in comparison with the RMI.

Total nucleated cell counts in the spleen (Fig. 7) and femur (Fig. 8) showed clear decreases in cell numbers and 'U'-shaped responses at 45 mg/kg busulphan. In each case the reduction in cell count was maximal at day 3 after dosing. With animals given 35 mg/kg busulphan, total nucleated cell counts in the spleen were within

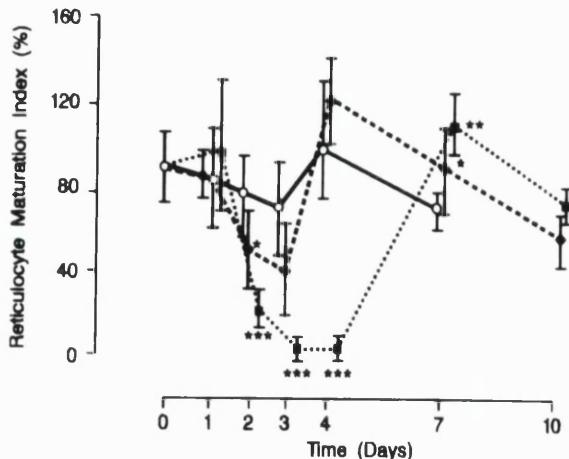


Fig. 6. Reticulocyte maturation index counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; ** p <0.01; *** p <0.001.

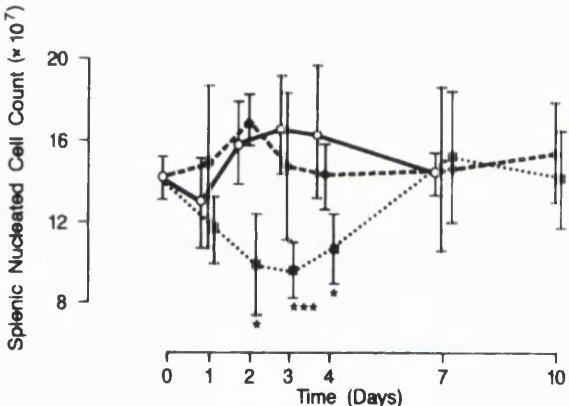


Fig. 7. Splenic nucleated cell counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; *** p <0.001.

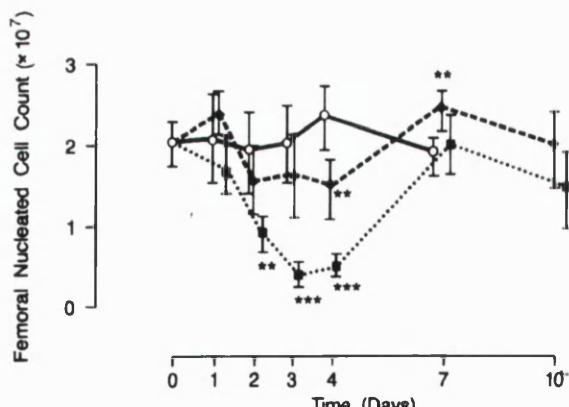


Fig. 8. Femoral nucleated cell counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. ** p <0.01; *** p <0.001.

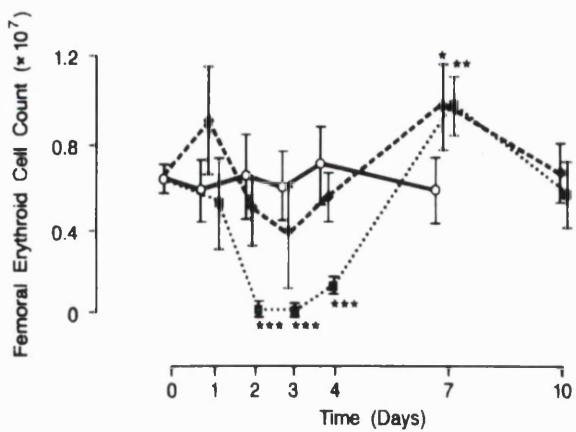


Fig. 9. Femoral erythroid cell counts in female B6C3F₁ mice given busulphan at 35 and 45 mg/kg intraperitoneally on day 0. —○—, control; —◆—, 35 mg/kg; —■—, 45 mg/kg. Each point represents the mean of values from five animals, \pm SD. * p <0.05; ** p <0.01; *** p <0.001.

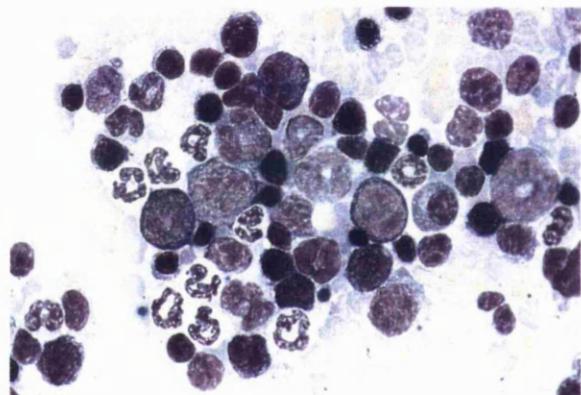


Fig. 10. Romanowsky stained bone marrow smear from an untreated (day 0) control mouse. Total nucleated cell count = $2.18 \times 10^9/l$; myeloid: erythroid ratio = 1.35:1. Magnification $\times 1000$.

control values throughout the experimental period. However, the nucleated cell count in the femoral sample at 35 mg/kg was reduced in number.

Differentiation of the femoral cells demonstrated that all cell types were affected in a dose-dependent manner. Femoral erythroid cells (Fig. 9) were almost completely depleted at day 2 post-dosing. This result finds a parallel with values for the percentage HFR (Fig. 5), and the RMI (Fig. 6). Myeloid and lymphoid elements of the femoral marrow were at their lowest at days 3 and 4 after dosing. All marrow and spleen counts in treated mice returned to normal or supranormal values by day 7 after dosing.

The appearance of Romanowsky stained bone marrow smears are illustrated from an untreated day 0 control animal (Fig. 10), and from the mice treated with 45 mg/kg busulphan (Figs. 11–13). At day 2 in a treated animal (Fig. 11), the cellularity was markedly decreased, and no primitive myeloid or erythroid forms were seen. At day 4 (Fig. 12), cellularity was still

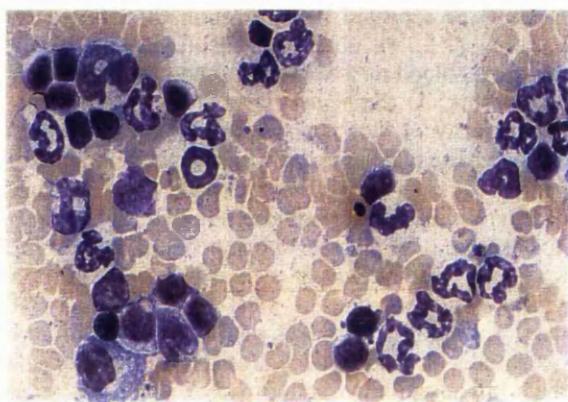


Fig. 11. Romanowsky stained bone marrow smear from a mouse 2 days after intraperitoneal treatment with 45 mg/kg busulphan. Total nucleated cell count = $0.92 \times 10^9/l$; myeloid: erythroid ratio = 62.00:1. Magnification $\times 1000$.

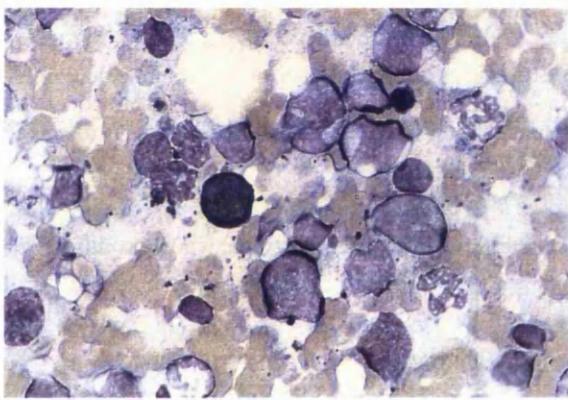


Fig. 12. Romanowsky stained bone marrow smear from a mouse 4 days after intraperitoneal treatment with 45 mg/kg busulphan. Total nucleated cell count = $0.45 \times 10^9/l$; myeloid: erythroid ratio = 6.24:1. Magnification $\times 1000$.

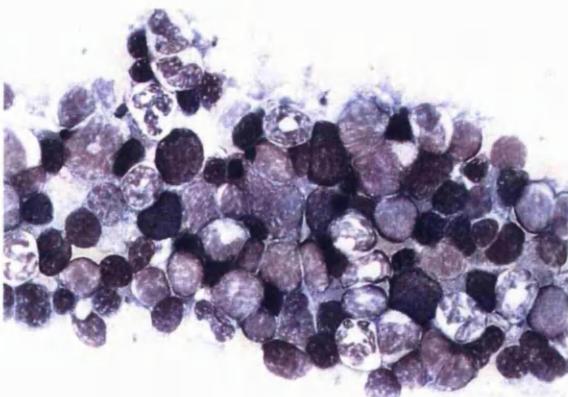


Fig. 13. Romanowsky stained bone marrow smear from a mouse 7 days after intraperitoneal treatment with 45 mg/kg busulphan. Total nucleated cell count = $2.45 \times 10^9/l$; myeloid: erythroid ratio = 0.53:1. Magnification $\times 1000$.

profoundly reduced, but almost all cell types were early forms, clearly showing a resurgence of haematopoiesis. By day 7 (Fig. 13) the cellularity of the marrow had returned to normal, but there was still an excess of immature forms compared with the untreated marrow (Fig. 10).

A significant decrease in platelet count was seen in mice dosed at 45 mg/kg busulphan, but only at day 3 after dosing. The administration of busulphan at 35 mg/kg produced no significant changes in platelet numbers. No treatment-related changes were seen in values for MPV or PDW.

Discussion

In attempting to establish a dosing protocol in B6C3F₁ mice, single doses of busulphan in acetone vehicle were first administered at levels from 10 to 60 mg/kg (Experiment 1). At 50 mg/kg and above, all animals died. At two weeks after treatment at dose levels from 10 to 45 mg/kg, all cellular elements were affected (Table 1). Leucocyte types showed a dose-related depression in numbers with the exception of lymphocytes, which appeared to be stimulated at low busulphan dosages. At high busulphan dose levels, MCV, MCHC, and hypochromic and macrocytic erythrocytes were affected, as were reticulocyte and platelet counts (Andrews et al. 1992). To investigate these changes in greater detail, a repeat dose study was carried out (Experiment 2) at levels from 10 to 40 mg/kg busulphan, as the first experiment had shown that no anaemia (i.e. profound alteration in Hb, RBC or HCT) occurred at 40 mg/kg (Table 1). The dosing regimen of Experiment 2 followed in outline the six-week protocol of Morley and Blake (1974a) where four doses of the drug were administered at fortnightly intervals. The timing of the two sampling time points at days 14 and 42 in Experiment 2 were based on the 14 day post-dosing period used in Experiment 1, and on the 6-week post-dosing period used by Morley and Blake (1974a) during which time mice were allowed to recover from the acute effects of busulphan. However, it was appreciated that early blood changes may have occurred and resolved during the immediate 14 day period following dosing. For this reason, Experiment 3 examined peripheral changes during the first 10 days after single doses of busulphan administered at the highest tolerated dose (45 mg/kg), and at an intermediate level (35 mg/kg). In order better to understand the peripheral blood changes taking place, femoral cell counts were also performed in Experiment 3, with the differentiation of femoral cells into myeloid, erythroid and lymphoid lineages. Spleen cell counts were also included in Experiment 3 as it is well known that extramedullary erythropoiesis is important in the mouse (Boggs et al. 1969; Bozzini et al. 1970; Pugsley et al. 1978).

The haematological changes seen at day 14 after a single dose of busulphan (Experiment 1, Table 1) were similar in many ways to those evident at 14 days in the

repeat dose study (Experiment 2, Table 2). Although the haematological effects in these experiments differed in degree, the same parameters were affected, i.e. there was a depression of all cellular elements, increased hypochromia and macrocytosis, and a non-significant elevation of reticulocytes. In addition, the MPXI was elevated in both experiments.

There are many reports of acute haematological toxicity in rodents treated with cytotoxic agents, and for this reason it was important to identify the immediate post-dosing effects of busulphan in the B6C3F₁ mouse. Results of other workers indicate that, depending on the dose of cytotoxic drug administered, haematological changes in rodents are either reversible, or progressive and result in the eventual death of the animal. Elson (1955) reported that albino rats given 20 mg/kg of busulphan intraperitoneally, died at 10–12 days post-treatment following aplasia and acute haemorrhage, usually into the stomach. Similarly, Santos and Tutschka (1974) showed that a single dose of 35 mg/kg busulphan administered to LEW rats caused a progressive drop in erythrocyte, granulocyte and lymphocyte numbers, with the death of animals at 13 days. Notable in the studies of Santos and Tutschka (1974) and Elson et al. (1958) was a biphasic progression of cytopenia. The response showed that after an initial depression, there was a resurgence of counts at days 3–5 post-treatment. However, at the levels of busulphan used, namely 20 mg/kg (Elson et al. 1958) and 35 mg/kg (Santos and Tutschka 1974), there was a subsequent relapse with eventual aplasia. At a lower busulphan dose level of 13 mg/kg, Elson et al. (1958) showed a recovery from the second relapse, whereas at 10 mg/kg, Santos and Tutschka (1974) reported no second relapse. A similar pattern of events without a second relapse was also described by Joyce et al. (1979) who studied the effect of granulopoiesis in C57BL X DBA/2 F₁ mice given 200 mg/kg cyclophosphamide intra-peritoneally. After the nadir of the peripheral neutrophil counts at 4 days post-treatment, there was a recovery to control levels by day 8, and then a considerable overshoot in the cell number.

In Experiment 3, the pattern of events observed in the bone marrow and peripheral blood showed the immediate toxic effect of busulphan on erythropoiesis (Figs. 1–13). Then, approximately 24 h after this response, myelopoiesis reached a nadir, and lymphoid cells were similarly depressed. The subsequent recovery of peripheral elements to normal levels was seen to lag behind the recovery of the marrow cells, an effect particularly evident in the erythroid cell population. However, an unexpected finding was that there were no clearly evident changes in platelet counts or MPV. This result was unanticipated as chemotherapy is well known to affect the platelet count in man with a depression in platelet number being seen after 7–10 days (Bessman 1982). The effects noted in Experiments 1 and 2 indicate that a depression of platelet counts may take longer to become established in the mouse, in comparison with man.

After the repeated administration of busulphan in Experiment 2, we observed consistent alterations in peripheral blood measurements at both 14 and 42 days post-dosing. At the highest dose levels (30–40 mg/kg) the majority of blood parameters showed significant changes. The platelet count was depressed in all treatment groups at 14 and 42 days post-treatment. Changes at several dose levels were also noted in MCV, MCH, and in percentage macrocytic and percentage hypochromic cells. Although some dose-related parameters showed a more significant effect at 42 days than at 14 days, in general the changes were of the same order of magnitude, or were reduced, at day 42. We consider that this consistent pattern of changes in many parameters (including some 'non-standard measurements') at both time points, indicates that it is possible to characterise busulphan-induced haematological changes with a considerable degree of accuracy.

Morley and Blake (1974a,b) and Morley et al. (1978) summarised the haematological changes evident in clinically well mice at 2–12 months after a repeat-dose treatment regimen with busulphan. During this time there were few changes in peripheral blood, with little or no effects on Hb levels or platelet counts, and only a moderate decrease in neutrophil count. However, the principal and most marked alteration was to the bone marrow, where a 57% decrease in total cell number was seen. Boyd et al. (1986), using the same dosing regimen with busulphan as Morley and his co-workers, were similarly unable to demonstrate significant differences in HCT or granulocyte counts in treated Swiss mice. However, Iderha et al. (1984) found significant decreases in RBC, WBC, granulocytes, lymphocytes and monocytes at 30 days post-treatment with busulphan in Swiss mice. A significant depression in HCT was described by Jelkmann and Bauer (1980) in NMRI mice after 6 weekly i.p. injections of busulphan at 5 mg/kg, a regimen found to give comparable long-term effects to those reported by Morley and Blake (1974a). Also, at 60 days after treating C3H/HeJ mice with four biweekly doses of 5 mg/kg busulphan i.p., Fitchen and Cline (1980) reported depressed lymphocyte counts.

The present experiments were carried out to establish a dosing regimen with busulphan in female B6C3F₁ mice based on the protocols of Morley and Blake (1974a). In their report of late bone marrow failure in Swiss mice, these workers described a treatment regimen where doses of 20, 20, 20 and 10 mg/kg busulphan were administered at two-weekly intervals over a period of six weeks. This dosing procedure was based on a previously determined LD₅₀ for busulphan of 30 mg/kg (Morley and Blake 1974a). Iderha et al. (1984) and Boyd et al. (1986) followed identical dosing regimens with busulphan in Swiss mice to those reported by Morley and Blake (1974a). Other workers have reported the use of lower levels of busulphan to induce myelotoxic effects, for example Jelkmann and Bauer (1980) gave 5 mg/kg weekly to NMRI mice for six weeks, and Fitchen and Cline (1980) administered 5 mg/kg on four occasions at two-weekly intervals to C3H/

HeJ mice. Freireich et al. (1966) reported that the 'toxic' (LD_{10}) dose of busulphan in Swiss and BDF₁ strain mice was 15 mg/kg. This compared with the 'toxic' level in rats which had been found in earlier unpublished studies by Schmidt to be 3.7 mg/kg (see Freireich et al. 1966). Doses between 10 and 15 mg/kg busulphan were administered to albino rats by Elson (1955) and Elson et al. (1958) after studies had determined the LD_{50} to be 20 mg/kg. However, Santos and Tutschka (1974) gave LEW strain rats a single dose of 35 mg/kg busulphan, having first established that the LD_{50} in this rat strain was 25.5 mg/kg. These results would therefore appear to show that there is variation in the response of different strains of mice and rats to the toxic effects of busulphan. Furthermore, our present studies appear to indicate that B6C3F₁ mice were able to tolerate rather higher levels of drug than is reported for other mouse strains in the literature.

The earlier studies by Morley and his co-workers (Morley and Blake 1974a,b; Morley et al. 1978), and other investigators were limited by the haematological techniques then available. Serial bleeding from the orbital sinus or tail vein only provided sufficient blood for a haematocrit estimation (Morley and Blake 1974a), and where full blood counts were performed, these involved haemocytometer counting and differential counts on stained smears (Fitchen and Cline 1980). Platelet counting was not initially available as a 'standard' haematological parameter, and reticulocyte enumeration was time consuming and imprecise. The recent availability of reliable flow cytometric-based haematology analysers (Ross and Bentley 1986; Coulet and Bezou 1990) which require low blood volumes, has made this new technology attractive for the analysis of laboratory animal blood samples. Now that software and hardware modifications have been evaluated (Takami and Sakata 1990; Davies and Fisher 1991; Fuchs and Eder 1991) for the analysis of blood from some non-human species, it has become possible to validate this new technology in an experimental setting. In this way we have been able to study the effects of busulphan in the B6C3F₁ mouse from the viewpoint of the standard haematology profile, and also using a further selection of 'non-standard parameters', totalling 26 in all. These values have also been correlated with the activity of the bone marrow and spleen.

The process of erythrocyte sizing and intracellular haemoglobin concentration measurements, carried out by the Technicon H*1, employs a technique involving laser light scattered at two angles following isovolumetric spherification using sodium dodecyl sulphate (Tycko et al. 1985). Errors innate in earlier technology (e.g. the underestimation of cell volume due to excessive light scatter by hypochromic erythrocytes, and the interferences by MCHC with measurement of MCV made by electronic impedance) are now eliminated and the isovolumetric spherification pretreatment gives results which compare to reference methods. It is hoped that MCHC measurement has now returned to clinical usefulness after a period of disrepute (Mohondas 1991). By setting

thresholds on the volume and haemoglobin distribution histograms, the assessment of microcytosis, macrocytosis, hypochromia and hyperchromia can be objectively quantified. Similarly, anisocytosis and variation in intracellular haemoglobin ('anisochromasia') are given numerical values which relate to the coefficient of variation of volume distribution, and the standard deviation of haemoglobin concentration, respectively.

However, in spite of such improvements, it has been observed that the H*1-calculated MCHC can be significantly greater than the (measured) intracellular haemoglobin concentration in iron deficiency patients (Bentley et al. 1989). This difference was also seen in the present studies in mice which are also microcytic and hypochromic, as compared to normal human erythrocytes; in many cases 'correlation error' flags were generated by the H*1, indicating a MCHC-CHCM difference of more than 1.5 g/dl. The reasons for this are not clear and Bentley et al. (1989) speculate that it might be 'an inherent property of the H*1'.

The anaemia, or drop in RBC, HCT and Hb values, seen at the highest busulphan treatment levels in the present studies, was in all cases macrocytic and hypochromic. That is, there was an increase in MCV, and a less convincing decrease in MCHC, but a clearly marked increase in macrocytic and hypochromic cells. Such an increase has been noted to parallel closely a rise in reticulocyte counts in rabbits (Davies, D. T., personal communication). However, we have examined the results from a large number of 'unscheduled deaths' in a separate long-term experiment involving busulphan-treated mice. In this study, a wide range of reticulocyte counts was seen, but we have been unable to find a correlation between reticulocytes and macrocytes, either when percentage or absolute numbers were examined. Moreover, the presence of an increased MPXI may suggest another explanation. The assessment of leucocyte peroxidase and cell size together, using sophisticated, species-specific software, enables the H*1 to differentiate leucocyte populations. Neutrophils are large, peroxidase-dense cells (Hayhoe and Flemans 1992) and the measurement of a high MPXI by the H*1 may be associated with neutrophil immaturity (Bentley et al. 1987). Acquired myeloperoxidase deficiency has been reported in a case of aplastic anaemia by Bizzaro et al. (1988), and a drop in MPXI has been associated with busulphan treatment (Froom et al. 1989). However, Gulley et al. (1990) demonstrated that a high MPXI may be caused by undetected megaloblastic anaemia, and Froom et al. (1989) reported a dose-dependent escalation of the MPXI following hydroxyurea therapy, which returned to normal after the cessation of treatment, whereupon the MCV became elevated. Hydroxyurea is known to interfere with ribonucleoside diphosphate reductase activity, and the drug thereby blocks DNA synthesis, as occurs in vitamin B12 deficiency. As busulphan acts as a cytotoxic agent in a comparable way to hydroxyurea, the busulphan-induced change to MPXI may be the result of mechanisms similar to those known to occur with hydroxyurea.

Hewitt and Reardon (1991) have observed that an apparent increase in neutrophil myeloperoxidase recorded by the Technicon H6000 is associated with a low haemoglobin concentration, and postulated that the pseudo-peroxidase activity of Hb competes for substrate. We have examined our data for evidence of such an association and found, in the Repeat Dose Study (Experiment 2) at 14 days post-treatment, where a wide range of MPXI values was present, a negative correlation ($r = -0.73$) between Hb and MPXI for all samples, irrespective of the treatment given. Therefore, the possibility that a low Hb content of the sample contributed to increased MPXI values cannot be dismissed. Nevertheless our data also shows that a negative correlation of almost equal magnitude ($r = -0.62$) existed between the neutrophil count and MPXI, indicating that MPXI measurements reflect properties of the neutrophil. It should be borne in mind that the relationship between low Hb and low neutrophil count ($r = 0.52$) is itself treatment related, so that any association between raised MPXI and low Hb may indeed be of a secondary nature.

In addition to a leucopenia affecting granulocytes, lymphocytes and monocytes, a significant depression of the LUC count was seen at high dosage levels in Experiment 2 at 42 days after repeat dosing with busulphan (Table 3). The LUC population, in blood counts which are normal, may for convenience be added to the lymphocyte population (Kershaw et al. 1987; Davies and Fisher 1991) since the stained films prepared from such samples do not reveal any abnormal mononuclear cells. However, Kinsey and Watts (1988) found that correlations with manual differentials were improved significantly if half the LUC population was assigned to the monocyte count in leucopenic patients. Increases in LUC count may indicate impending relapse in acute leukaemia patients (d'Onofrio et al. 1987; Kline et al. 1989) or the recovery of the bone marrow following chemotherapy (Kinsey et al. 1988) and investigation of this parameter may be of interest in the subsequent progression of experimental busulphan-induced lesions.

Platelet counts showed a progressive and marked dose-related depression in all busulphan-treated animals in the present studies, following repeat dosing (Experiment 2) and at 14 days after a single dose (Experiment 1), but not during the shorter sequential sampling study (Experiment 3). However, with the exception of elevated values in all dose groups in the single dose study (Experiment 1), there was little effect on MPV. Bessman (1985) reported the association of platelet count and MPV in a variety of disorders. There was a non-linear inverse relationship between the two parameters in man in health, but *inter alia*, MPV is inappropriately low in aplastic anaemia patients, and in those treated with myelosuppressive drugs. In chemotherapeutic regimens, platelet count and MPV may fall simultaneously and, following recovery, the rise in platelet count is preceded by 1-2 days by an increase in MPV, which in turn is prefaced by an increase in megakaryocyte ploidy (Bessman 1982). The only

suggestion of an association between platelet count and MPV in the present study was in Experiment 2 at 42 days after repeat busulphan administration, where there was evidence of decreased platelet numbers and reduced MPV at high drug levels.

Automated analysis of reticulocytes by flow cytometry using thiazole orange (Mechetner et al. 1991) or acridine orange and immunofluorescence (Seligman et al. 1983; Nehls et al. 1991) has been available for the analysis of human and animal blood for some time. Although conventional flow cytometers are extremely versatile, they require careful calibration and setting up, and manual sample preparations may be necessary with lengthy preincubation periods. However, the use of dedicated reticulocyte analysers now enable the routine analysis of reticulocyte samples from experimental animals to be carried out (Takami and Sakata 1990; Fuchs and Eder 1991). Furthermore, in addition to performing a total reticulocyte count, reticulocyte analyses are able to quantitate the degree of maturity of specific reticulocyte fractions (Nagao et al. 1990). The reticulocyte response has thus been quantified in man (Cavill 1990) and the rat (Eder and Wiemer 1987) in response to erythropoietin, and bone marrow transplantation (Davis et al. 1989, 1992). The last two studies used a maturation index and the quantitation of highly fluorescent reticulocytes as early markers of regenerating erythropoiesis. Tatsumi and Tsuda (1991) describe the use of a Sysmex R-1000-derived reticulocyte maturation index (RMI), where $RMI = [(MFR + HFR)/LFR] \times 100\%$. This index, although appearing to be a very sensitive indicator of bone marrow erythropoiesis was probably slightly less sensitive than HFR alone, in that the dispersal of data was greater when expressed as the RMI. However, in Experiment 3, both HFR and RMI accurately mirrored the very low levels of bone marrow erythroid activity at 2 days after busulphan dosing and heralded the regenerative responses and supranormal values at 4 days (35 mg/kg busulphan) and 7 days (45 mg/kg busulphan). The total reticulocyte numbers for these animals on these occasions were seen only to overlap the normal range.

In conclusion, we have demonstrated that for the female B6C3F₁ mouse, a relatively high dosage of busulphan (40 mg/kg) may be administered on four fortnightly occasions with no treatment-related mortality evident at 6 weeks after the dosing period. A characterisation of the myelotoxic effect of busulphan was carried out, using standard flow cytometry-based haematology analysers, which had been programmed or modified for use with mouse blood. There was an initial acute cessation of marrow activity, notably erythropoiesis, which then recovered to slightly subnormal levels within the first two weeks, and thereafter a persistent reduction in red cell, leucocyte and platelet numbers was evident. The measurement and reporting of 'non-standard parameters', such as MPXI and HFR, was therefore considered to be useful in these investigations on the reaction of the haemopoietic system in the mouse. Examination of these 'non-standard para-

meters' may therefore be of value in other toxicological investigations involving cytotoxic agents and also in studies on the toxicity of drugs of other classes.

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Original Article

Long-term Effects of Busulphan on Lymphocyte Subpopulations in Female B6C3F₁ Mice

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Abstract. Female B6C3F₁ mice (12–14 weeks old) were dosed with busulphan (BU) by the intraperitoneal route at a range of dose levels between 10 and 40 mg/kg on four occasions at 14 day intervals. Six weeks after the final dose, mice were given normal drinking water or drinking water containing chloramphenicol succinate (CAP.S) at 4 mg/ml. Animals were killed at eight timepoints after the final BU dose, the final samples being taken at day 485 or 497. A range of haematological parameters were determined and lymphocyte subsets analysed. The inclusion of CAP.S in the drinking water did not affect the changes to haematological parameters or lymphocyte subsets induced by BU. Lymphocyte count was reduced to 51%–66% of control values in animals receiving 40 mg BU/kg on all occasions except day 85. However, the lymphocyte count was increased to 107%–143% of control values in animals receiving 10 mg BU/kg on all occasions except day 85. The large unstained cell (LUC) count was reduced to between 25%–47% of control values in animals receiving 40 mg BU/kg on all occasions, however, the LUC count was not increased at low BU dosage. At day 485/497, B lymphocyte counts were reduced to 48% ($p<0.01$), 64% (NS) and 55% ($p<0.05$) of control values in mice treated with 40, 33 and 25 mg BU/kg, respectively. T lymphocyte counts were not affected by BU treatment. We have, therefore, demonstrated in the present study that administration of BU at high levels to the B6C3F₁ mouse induces a long-lasting

and specific depletion of peripheral B lymphocytes; also, BU at low dose levels causes a mild but persistent lymphocytosis.

Keywords: Busulphan; Chloramphenicol succinate; Lymphocytosis; Lymphocytopenia; Large unstained cells; Mouse

Introduction

In several studies conducted in the 1970s, Morley and co-workers (Morley and Blake 1974ab; Morley et al. 1975) established a model of chronic hypoplastic marrow failure in Balb/c and Swiss mice by treating them with high intraperitoneal doses of the alkylating agent busulphan (BU; also known as myleran). Morley was able to assert that stem cell damage due to BU treatment predisposed the mice to chloramphenicol-induced marrow aplasia in a predictable fashion (Morley et al. 1976). However, Robin et al. (1981) appear to have been unsuccessful in their attempt to repeat this work in CAF₁ mice and as late as 1986 it could still be stated that 'no reliable model for the prediction of aplastic anaemia exists' (Vincent 1986). Nevertheless, a murine model of aplastic anaemia (AA) would be of great value in the preclinical development of new medicines. It was with this in mind that we set out to study Morley's experimental work with the intention of demonstrating the bone marrow lesion in the female B6C3F₁ mouse, a strain commonly used in preclinical toxicity testing. We

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present here unexpected findings on lymphocytes which further characterise the long-term haematological lesion induced by BU.

Materials and Methods

1. Animals

Female B6C3F₁ mice (Glaxo Group Research Ltd), 12–14 weeks old, were housed in groups of 5 or 6, bedded on wood shavings, and given diet (Rat and Mouse No. 1, SDS Ltd., Witham, Essex) and drinking water ad libitum. A temperature of 22°C ± 1°C was maintained, with a relative humidity of 45%–70% and a 12:12 h light:dark cycle (lights on at 06.00 h). Animals were acclimatised for at least 5 days before the start of the experiment and were observed once daily for signs of ill-health, but more frequently after each BU dose.

2. Dosing

Busulphan. BU (Sigma Chemical Co. Ltd, Poole, Dorset) was dissolved in acetone at a concentration of 16.7 mg/ml. The BU solution was administered by intraperitoneal injection after dilution with distilled water (Water for Irrigations, BP) to give a constant dose volume of 10 ml/kg bodyweight. Control mice were given acetone with distilled water at a dose volume of 10 ml/kg.

Chloramphenicol. Chloramphenicol succinate (CAP.S), (Sigma) was dissolved in distilled water and administered in the drinking water at a concentration of 4.0 mg/ml.

3. Experimental Design

A total of 336 mice were randomly assigned to six groups of 36 (groups 1 to 6) and six groups of 20 (groups 7 to 12). Animals in groups 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12 were dosed on four fortnightly occasions with BU at 0 (controls), 10, 18, 25, 33 and 40 mg/kg, respectively. Six weeks after the fourth dose of BU, on day 85, animals in groups 1 to 6 were given CAP.S in drinking water ad libitum whereas those in groups 7 to 12 were given drinking water without the antibiotic.

4. Haematological Measurements

Animals were killed by CO₂ overdose and K₂EDTA anticoagulated blood samples were taken from the posterior vena cava. Samples were taken on days 0, 42 (after the last BU dose), 85 (on the first day of CAP.S administration), 140, 177, 210, 252, 384, 485 or 497. For logistical reasons, groups 1 to 6 were bled on day 485, and groups 7 to 12 on day 497. Blood was analysed

using a Bayer H*1 haematology analyser with mouse-specific software (Bayer Ltd, Newbury, Berks, UK) and a Sysmex R-1000 reticulocyte analyser (Sysmex UK Ltd, Milton Keynes, Bucks, UK).

In the analysis of leucocytes by the Bayer H*1, neutrophils, lymphocytes, monocytes and eosinophils are differentiated after staining for peroxidase content. When these stained cells pass through the flow cell of the analyser, staining intensity is assessed according to tungsten light absorption. Simultaneously, the size of cells is assessed by forward light scatter. Cluster analysis consists of the resulting patterns being compared to an archetype for leucocytes of the particular species in question. A small proportion of cells fall into an area on the cytogram indicating greater cell size than most lymphocytes, but having lower peroxidase activity than monocytes. Such cells are classified as 'large unstained cells' (LUC). In human blood, it has been shown that assigning half the LUC count to lymphocytes and half to monocytes produces a differential leucocyte count very close to manual (visual) examination of the Romanow-sky-stained smear (Kinsey and Watts 1988).

5. Lymphocyte Subset Analysis

Day 384. Blood (0.5 ml) anticoagulated in 0.106M trisodium citrate (9 vol. blood:1 vol. citrate) was added to 3.0 ml ammonium chloride for erythrocyte lysis and centrifuged at 350 g for 10 min at 4°C. The pellet was then washed in RPMI + HEPES buffer with 5% fetal calf serum and 0.05% azide, and centrifuged at 350 g for 5 min at 4°C. The resuspended pellet was subdivided into three aliquots which were treated with Caltag antibodies (Bradsure Biologicals Ltd, Loughborough, Leics, UK) as follows: (1) rat anti-mouse L3/T4 (CD4)-tricolor (Caltag catalogue number RM2400; 2 µl per 50 µl cell suspension) and rat anti-mouse Ly2 (CD8a)-fluorescein isothiocyanate (FITC) (cat. no. RM2200; 5 µl per 50 µl cell suspension); (2) goat anti-mouse IgG (H+L)-FITC (cat no. M30000; 2.5 µl per 100 µl cell suspension); (3) RPMI only, for autofluorescence control. After incubation for 30 min at 4°C, three further washes were performed as before. Samples were finally reconstituted in RPMI for two-colour analysis using a Coulter EPICS flow cytometer (Coulter Electronics, Luton, Beds, UK).

Day 485 and 497. EDTA blood (0.5 ml) was treated with RPMI + HEPES buffer as before, but the washed pellet was treated with distilled water to lyse the erythrocytes. After antibody concentration had been optimised by titration, three-colour analysis of Pan T, CD4 and CD8 cells, and two-colour analysis of Pan T and B cells were performed simultaneously using a Becton Dickinson FACScan (Becton Dickinson, Cowley, Oxon, UK). Three-colour analysis was with mouse-anti mouse Thy1.2 (Pan T)-phycoerythrin (PE) Caltag catalogue number MM2000, rat-anti-mouse L3/T4 (CD4)-tricolor (cat. no. RM2400) and rat-anti-mouse Ly2 (CD8a)-FITC (cat. no. RM2200). Two-colour analysis was with

mouse-anti-mouse Thy1.2 (Pan T)-PE (cat. no. MM2000) and goat-anti-mouse IgG (H+L) (B cells)-FITC (M30000). Antibody concentrations used were: anti-Thy 1.2: 5 µl in 50 µl; anti-IgG: 2 µl in 50 µl; anti-L3/T4: 2 µl in 50 µl; anti-Ly2: 5 µl in 50 µl; incubations were for 60 min. Autofluorescence of normal samples was negligible in all cases. Isotype controls gave the following background levels of fluorescence: rat IgG 2b-FITC (for Ly2), 1.8%; rat IgG 2b-Tricolor (for L3/T4), 6.0%–6.4%; mouse IgG 2b-PE (for Thy 1.2), 0%; goat IgG-FITC (for B cells), 2.5%.

6. Statistical Analysis

Student's *t* test was used to compare treated and control groups at the same timepoint. Two-way analysis of variance was used to compare data on days 485 and 497.

Results

Statistical analysis of groups 1 to 6 (those receiving CAP.S in drinking water) against groups 7 to 12 (those given water without the antibiotic) revealed no effects on haematological parameters that could be ascribed to the administration of the drug. Therefore the data discussed below are pooled from each BU dose level (i.e. groups 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12).

Leucocytes

In animals receiving 33 and 40 mg BU/kg the total leucocyte count was reduced to 50%–80% of control values at all occasions after the start of BU dosing, with the exception of day 85 (Fig. 1). This was due in the earlier part of the study to a reduction of all leucocyte types. However, in animals receiving 10 mg BU/kg, an increase in leucocyte count was seen on all occasions from day 85 onwards, and on days 85, 252, 384 and 485/497 in animals receiving 18 mg BU/kg.

Lymphocytes

The most marked effect on the total leucocyte count was due to changes in the lymphocyte count (Fig. 2). In the group receiving 40 mg BU/kg, again with the exception of day 85, the lymphocyte count was reduced to 51%–66% of control values on all occasions. In animals receiving 33 mg BU/kg, a lowering of lymphocyte count was also seen (except on day 85) and ranged between 62% and 85% of control values. Elevations of the lymphocyte count were observed in mice receiving 10 mg BU/kg, and to a lesser extent at 18 mg BU/kg. In the 10 mg/kg group, counts ranged between 107% and 143% of controls from day 85 onwards, whereas in the 18 mg/kg group, counts were only elevated on days 85, 252 and 384, ranging between 123% and 133% of controls.

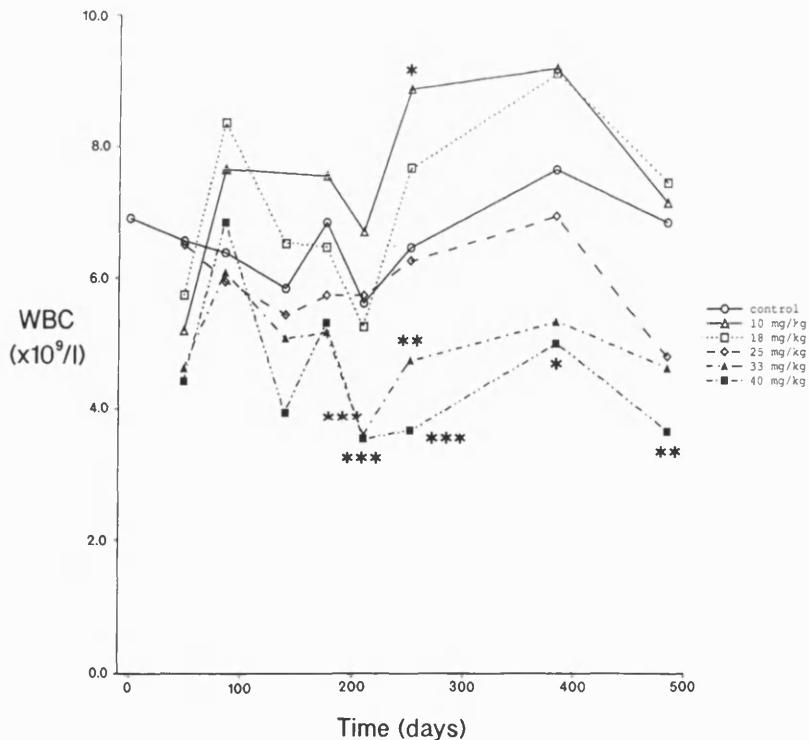


Fig. 1. Total leucocyte counts in female B6C3F₁ mice after four fortnightly i.p. doses of BU (dose levels shown in key). $n = 5$ on days 0, 42, 85 and 384; $n = 10$ on all other occasions (except day 485/497, where $n = 9$ in control and 10 mg BU/kg groups). * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$.

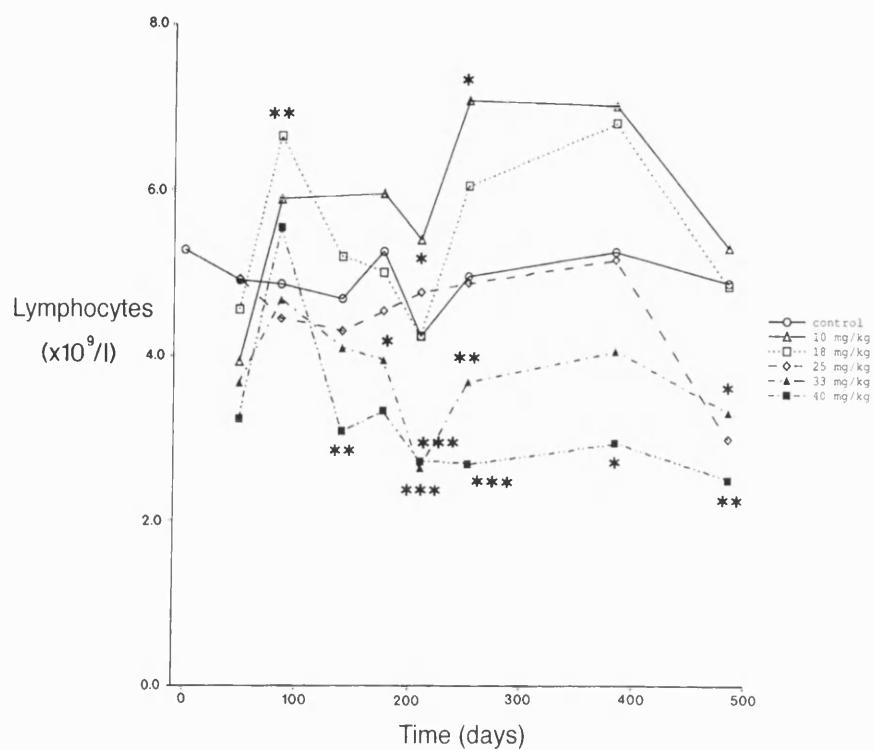


Fig. 2. Lymphocyte counts in female B6C3F₁ mice after four fortnightly i.p. doses of BU (dose levels shown in key). $n = 5$ on days 0, 42, 85 and 384; $n = 10$ on all other occasions (except day 485/497, where $n = 9$ in control and 10 mg BU/kg groups). * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$.

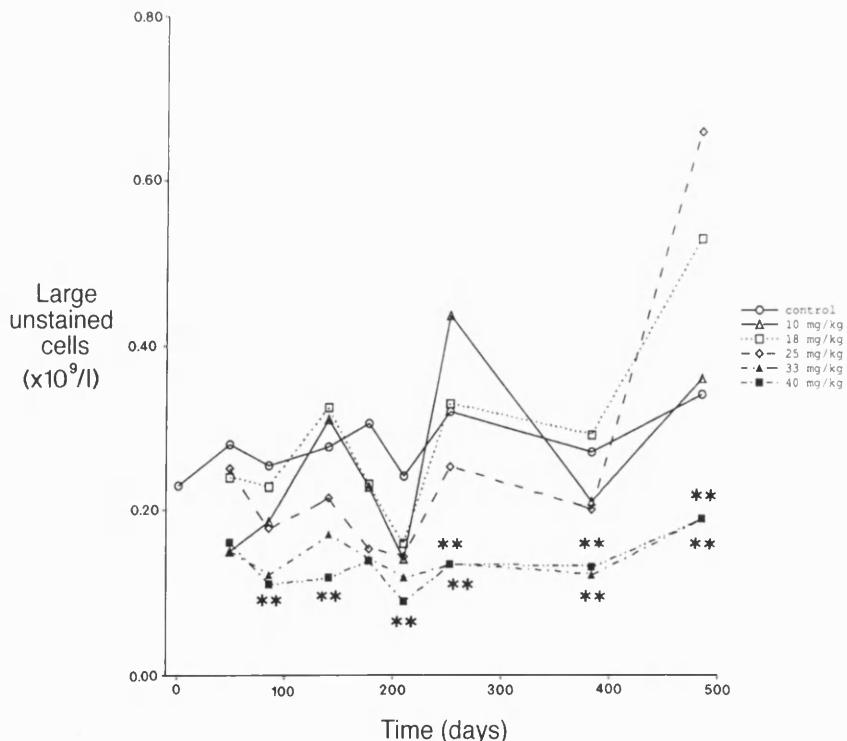


Fig. 3. Large unstained cell counts in female B6C3F₁ mice after four fortnightly i.p. doses of BU (dose levels shown in key). $n = 5$ on days 0, 42, 85 and 384; $n = 10$ on all other occasions (except day 485/497, where $n = 9$ in control and 10 mg BU/kg groups). ** $p = 0.01$.

Large Unstained Cell Counts

As with the lymphocyte counts, decreased numbers of LUC were seen in the 33 and 40 mg BU/kg groups (Fig. 3). The reductions were seen consistently in these two dosage groups from day 42 onwards, and were proportionally greater than the reductions in lymphocyte count, values ranging between 25% and 47% of control values in animals receiving 40 mg BU/kg, and between 31 and 53% of controls in the 33 mg BU/kg group. At the same time, a rising incidence of high individual values was observed with increasing time in the other groups in a non-dose-related fashion. However, the slight but sustained increase in cell numbers seen at the 10 mg/kg level for lymphocytes was not present in LUC counts.

Lymphocyte Subsets

Day 384. Preliminary examination of lymphocyte subtypes on day 384 revealed a selective effect on B cells alone (Fig. 4). Cells expressing IgG were reduced to 31% and 25% of control values in the 33 and 40 mg BU/kg groups, respectively. However, the proportion of unstained cells in this analysis was also considerably greater in these two groups. This was considered to be due in part to inadequate lysis of erythrocytes using ammonium chloride; therefore it is not possible on the basis of these data to state definitively that the effect was confined to B cells.

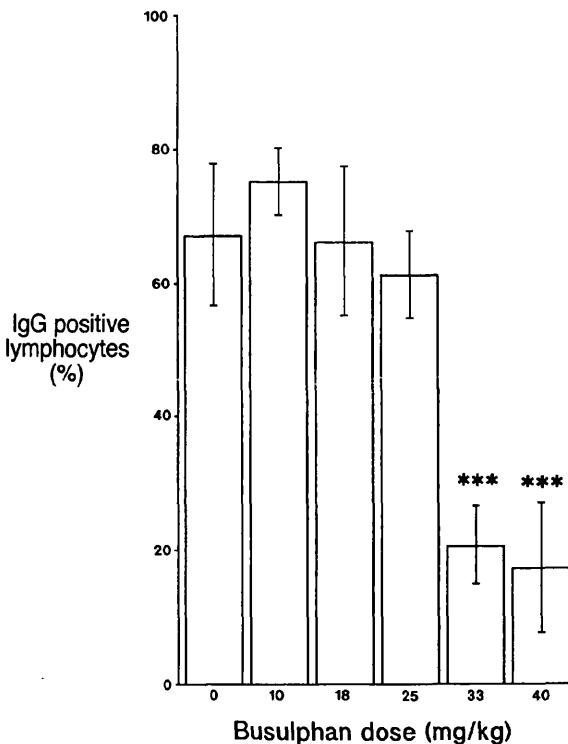


Fig. 4. Percentage IgG-positive lymphocytes in female B6C3F₁ mice on day 384. Four fortnightly doses of BU were administered on day 0, 14, 28 and 42. $n = 5$; bars denote mean values, \pm SD. *** $p = 0.001$.

Days 485 and 497. The measurements performed at day 384 were repeated at the end of the study on days 485 and 497 and the data from the latter two occasions were pooled. On this occasion, a benchtop flow cytometer was available, allowing measurements to be made more quickly and conveniently. Lysis with distilled water gave a much more distinct mononuclear cell population, and the proportion of unstained cells in this analysis was much reduced, and consistent between groups. Two-way analysis of variance showed no significant differences between the results on days 485 and 497 for either IgG positive or Thy1.2 positive lymphocyte subsets.

The total lymphocyte counts are illustrated in Fig. 5. There were statistically significant reductions in the counts in animals treated with 25 and 40 mg BU/kg. The pattern of depression of B lymphocytes (Fig. 5) mirrored that seen in the total lymphocyte counts. A reduction to 48% of control values was seen in animals receiving 40 mg BU/kg ($p = 0.01$). In the 25 mg/kg group, counts were depressed to 55% of controls ($p = 0.05$), and at 33 mg/kg, to 64%, but this last result was not statistically significant. B lymphocyte counts were increased to 116% of controls in the lowest dose group (10 mg BU/kg), but again, this result did not achieve significance. Mean values for CD4+ and CD8a+ lymphocytes in BU-dosed animals were not significantly different from controls.

Excluded from the analysis of day 485 and 497 results were two animals with a marked leucocytosis, one a control animal not treated with BU, and one which had

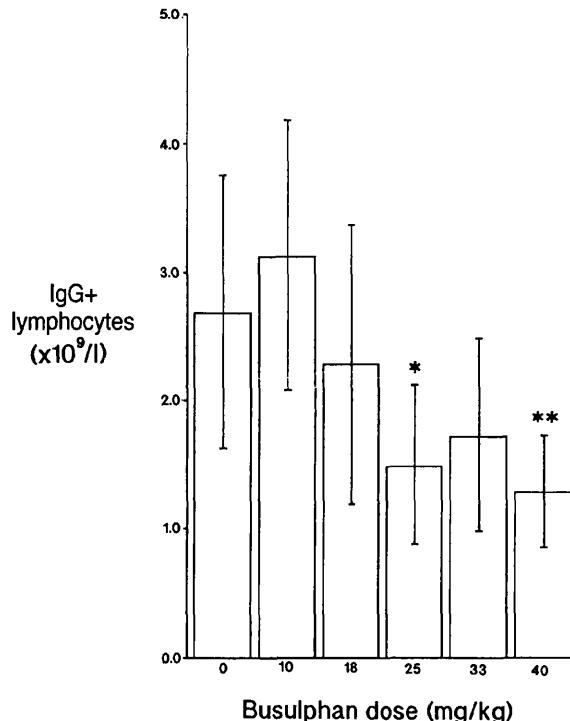


Fig. 5. IgG positive lymphocytes in female B6C3F₁ mice on day 485/497. Four fortnightly doses of BU were administered on days 0, 14, 28 and 42. $n = 10$ (except control and 10 mg BU/kg groups, where $n = 9$); bars denote mean values, \pm SD. * $p = 0.05$; ** $p = 0.01$.

received 10 mg BU/kg. Microscopically, many leucocytes from these two animals possessed the characteristics of lymphoma cells.

Discussion

In preliminary work to establish the maximum tolerated dose levels of BU (Andrews et al. 1993), transient (over 10 days) reductions of the erythrocyte count, platelet count and the counts of various leucocytes including lymphocytes, were noted after single doses of 35 and 45 mg BU/kg. However, in that study, the lymphocyte count was not observed to be significantly depressed 14 days after a single intraperitoneal (i.p.) dose of BU of between 30 and 45 mg/kg, but there was a significant lymphocytosis after dose levels of between 10 and 25 mg/kg. A similar pattern was observed 14 days after four successive i.p. doses of BU, but the changes were not statistically significant. However, 42 days after this repeated dosing regime, a dose-dependent lymphocytopenia was observed at between 30 and 45 mg BU/kg. The reduction in lymphocyte count at 45 mg/kg was 57%.

In the present study, female B6C3F₁ mice exhibited a dual effect on the peripheral lymphocyte count after repeated doses of BU (Fig. 2). First, in animals receiving 40 mg/kg, a sustained depression of the lymphocyte count was seen throughout the study, except at day 85. This was first seen after the final dose of BU treatment (day 42), and averaged 51%–66% of control values in subsequent samples. This depression was also evident, but less marked in animals receiving 33 mg BU/kg. On the other hand, in animals receiving 10 mg/kg, a mild but consistent elevation of the lymphocyte count was observed. Analysis of subsets on days 384 and 485/497 (342 and 443/455 days after the final treatment with BU) demonstrated that these effects were primarily on B lymphocytes.

BU is an alkylating agent whose principal mode of haematotoxicity is myelosuppression. Granulocyte precursors are particularly sensitive to the inhibitory effects of the drug, although erythroid and thrombocytic cell lines may be affected adversely as a side effect of therapy. Thus, in humans, BU therapy was for many years the treatment of choice for chronic myeloid leukaemia (Hall and Tilby 1992). The drug was also widely used in polycythaemia vera and in myelofibrosis. Latterly its suppressive properties have been heavily used for myeloablative preparation for bone marrow transplantation (Beutler et al. 1995).

Most chemotherapy may be immunosuppressive and lymphocytopenia is an often noted accompaniment to treatment with alkylating agents (Brohee et al. 1991). However, unlike nitrogen mustards (Dunn, 1974) and cyclophosphamide (Braun and Harris, 1981; Tonner et al. 1988), BU is not considered to be immunosuppressive in humans, except at toxic doses (Mitchell 1988; Yeager et al. 1991).

BU was selected by Morley and his co-workers (Morley and Blake 1974b; Morley et al. 1975) for its ability to cause a long-standing depressive effect on colony-forming cells in mice. For this reason they proposed the use of the drug as the basis for an animal model for the study of aplastic anaemia (AA) (Morley and Blake 1974b). In AA, the principal lesion is a profound depression of erythropoiesis and myelopoiesis. In addition, however, reduced circulating numbers of B lymphocytes have been described (Morley et al. 1974). Sabbe et al. (1984) also found a lymphocytopenia in AA patients which was principally due to a depletion of B cells (but in addition an accompanying monocytopenia was found (Sabbe et al. 1984)). These patients demonstrated a reduced immune function which was correlated with depressed monocyte count. Gascon et al. (1986) have demonstrated that natural killer (non-B, non-T) cell number and activity are depressed in AA patients.

Animal studies have also demonstrated the selective toxicity of BU for granulocytic and erythroid precursors (Boggs and Boggs, 1980), but a number of studies have shown that lymphocyte numbers may be affected. At an early stage in the characterisation of the drug, BU was shown to cause a rapid reduction of lymphocytes in the marrow of albino rats, sustained for up to 26 days after a single oral dose of 15 mg/kg (Elson 1955; Elson et al. 1958). In rats (Santos and Tutschka 1974) and rabbits (den Ottolander et al. 1982) given single, high doses of BU, lymphocytopenia developed before to death. In mice, higher doses of BU are tolerated, and a number of studies have used a repeat-dose regimen. For example, transient suppression (up to 31 days post dose) of lymphocytes was noted in female CD2F₁ mice after a total of 25 mg BU/kg was given intraperitoneally (i.p.) five times daily, although neutrophil suppression was more marked (Kato et al. 1988).

In regimens with BU similar to that used in the present work, where animals are allowed to recover for one or two weeks between doses, prolonged effects on lymphocyte counts have been reported. A reduction in lymphocyte count of 63% was seen 60 days after four fortnightly i.p. doses of 5 mg BU/kg (Fitchen and Cline 1980). In female Swiss mice a sustained lymphocytopenia was seen up to 175 days after the cessation of fortnightly i.p. dosing with BU, totalling 70 mg/kg (Morley and Blake, 1974a). A significant depression of peripheral B (but not T) cell counts was observed four months after four fortnightly i.p. injections of BU totalling 60 mg/kg in female Balb/c mice (Pugsley et al. 1978). In female CF-1 mice, bone marrow lymphoid cells were depressed to 20% of control values six months after four weekly ip doses of 20mg BU/kg (Wathen et al. 1982). Natural killer cell activity has also been reported to be depressed in male CAF₁ mice up to four months after four fortnightly i.p. doses of 0.5 mg BU (Bhoopal et al. 1985). In experiments involving donor spleen, bone marrow and thymus from BU-treated mice, immunosuppressive effects were ascribed

not only to a depression of B cell number or function, but also to a subpopulation of T cells (Addison 1973; Dunn 1974).

Therefore, although a depressive effect on the lymphocytes of mice receiving high doses of BU has been described for four to six months post-dosing (Morley and Blake, 1974a; Pugsley et al. 1978), the mild, sustained increase seen in the present investigation following treatment at lower dosages was unexpected. Whereas the administration to mice of 10 mg BU/kg in this experiment caused an increase in lymphocyte count of up to 143% of control values, the effect was not seen in LUC. This would appear to indicate that the lymphocytosis consists of small lymphocytes. Although it is not possible to state with certainty from which lineage these cells are derived, there was a slight (but statistically insignificant) rise in both IgG positive and L3/T4 positive cells.

In contrast to the very prolonged lymphocytopenia described in the present study, and those referred to above where the response was for shorter periods, a sustained lymphocytosis has not been reported in association with BU therapy. A few experimental studies have noted a rebound in lymphocyte count (Elson 1955; Kato et al. 1988); in humans, rebound lymphocytosis may be seen after the cessation of chemotherapy (Borella et al. 1972). In general, however, it would appear that such a rebound is transient, and similar in character to the effect on erythropoiesis (Andrews et al. 1993). In humans, B cell proliferation has been observed after treatment with BU for polycythaemia vera (Papayannis et al. 1982; Koza and Babusikova 1988). The persistent generalised lymphadenopathy of HIV infection in man is characterised by a proliferation of B cells.

Persistent lymphocytosis is seen in humans in a variety of clinical conditions, indeed, idiosyncratic drug reactions may also be associated with subacute lymphocytosis (Beutler et al. 1995). However, these varied conditions are usually associated with the presence of morphologically atypical, activated lymphocytes, the presence of which will cause an increase in the LUC count. There was no associated increase in LUC count in the present study. An apparent lymphocytosis has been observed in paediatric patients with congenital hypoplastic anaemia, a condition sharing many diagnostic criteria with AA (Miale and Bloom 1975). It was noted in this study that predominantly small lymphocytes were involved.

A pattern of responses analogous to the present findings, in which leucocyte production is depressed at high dosage and stimulated at low dosage is seen when mouse haematopoietic precursor cells are treated *in vitro* with busulphan. At millimolar concentrations, a dose-related suppression of colony growth in response to GM-CSF (granulocyte-macrophage colony stimulating factor) is seen. However, at much lower (micromolar) concentrations, enhanced colony growth is seen, due to an increased clonogenic recruitment of cells normally unresponsive to GM-CSF (Irons and Stillman 1993).

Several mechanisms may therefore be considered responsible for the persistent lymphocytosis seen in the present study: first, a 'rebound' effect, similar to the erythroid 'overshoot' observed after a single dose of BU; secondly, a stimulatory effect specifically affecting immunocompetent B cells, and thirdly, a proliferative stimulus of BU at low dosage levels on a primitive cell type, morphologically indistinguishable from a small lymphocyte. It is not possible on the basis of the present findings to postulate which, if any, of these mechanisms may be occurring. Further work is necessary to clarify the nature of the effect.

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Original Article

Long-term Haematological Alterations in Female B6C3F₁ Mice Treated with Busulphan

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Abstract. Female B6C3F₁ mice (12–14 weeks old) were dosed with busulphan (BU) by the intraperitoneal route at dose levels between 10 and 40 mg/kg on four occasions at 14 day intervals. Six weeks after the final dose, mice were given normal drinking water or drinking water containing chloramphenicol succinate (CAPS) at 4 mg/ml. Animals were killed at eight timepoints after the final BU dose, the last samples being taken at day 485 or 497 and a range of haematological parameters measured. During the experiment, no differences could be detected between mice receiving BU and CAPS and those receiving BU alone. Animals surviving to the end of the study displayed moderate leucopenia but no reduction in marrow cellularity nor was any effect on erythropoiesis apparent. Lymphocyte numbers were reduced in peripheral blood and bone marrow, and splenic cellularity was also reduced. Increased mortality was seen in animals which had received 40 mg BU/kg. In the first four months after BU dosing, animals killed due to ill health were pancytopenic with hypocellular marrows; deaths that occurred thereafter were due to lymphoma.

Keywords: Busulphan; Chloramphenicol succinate; Haematological changes; Mouse

major adverse effects on the haematopoietic system (FAO 1988; IARC 1990). First, a commonly occurring, reversible, bone marrow suppression. This response is dose dependent and generally occurs within days of beginning antibiotic administration. The condition is typified by a reticulocytopenia and anaemia with occasional leucopenia and thrombocytopenia. There are no laboratory animal models of this response (Holt et al. 1998). The second adverse effect induced is marrow aplasia with pancytopenia (aplastic anaemia, AA). This idiosyncratic condition is unrelated to drug dose and is often irreversible and fatal (Yunis and Bloomberg 1964; Geary 1978; Yunis 1978). It is considered that in this second response a genetic element is involved giving rise to an individual predisposition (Morley et al. 1976; FAO 1988). In a series of investigations, Morley and his co-workers developed a laboratory animal model of chronic hypoplastic marrow failure (AA) in female Swiss or BALB/c mice by the intraperitoneal administration of the alkylating agent, busulphan (BU) (Morley and Blake 1974a, b; Morley et al. 1975, 1978). Using this mouse model, these investigators subsequently examined the idiosyncratic haemotoxicity of CAP, with the drug administered in the drinking water of BU-pretreated BALB/c mice (Morley et al. 1976); they concluded that BU pretreatment induced residual marrow damage and in this condition the marrow was more sensitive to the toxic effects of CAP.

Several investigators have examined CAP haemotoxicity in the BU/CAP mouse model of chronic hypoplastic marrow failure described by Morley et al. (1976), but their publications (Pazdernik and Corbett, 1980; Robin et al. 1981; Bhoopalam et al. 1986) give results which do not closely parallel those of Morley et al. Indeed, several reports published after that of Morley et al. (1976) put

Introduction

The broad spectrum antibiotic, chloramphenicol (CAP), is toxic to the bone marrow and in humans induces two

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forward the view that no suitable or reliable laboratory animal model of AA existed (Yunis 1978; Haak 1980; Appelbaum and Fefer 1981; Vincent 1986; FAO 1988). Nevertheless, a convenient animal model of AA would be of great benefit to biomedical research, particularly in studies to investigate the pathogenic mechanisms of the disease. Also, a suitable laboratory animal model would be of considerable value to the pharmaceutical industry in the preclinical development of new medicines.

With these considerations in mind, in an attempt to induce chronic hypoplastic marrow failure in the mouse, we have re-examined the experimental protocol of Morley et al. (1976) using the BU/CAP dosing regimen but employing the B6C3F₁ mouse, an F₁ hybrid commonly used in preclinical drug testing. We present here, comprehensive findings on peripheral blood changes, and the responses of the principal haematopoietic organs, in animals treated with BU following the protocol outlined by Morley et al. in their studies on AA (Morley and Blake 1974a, b; Morley et al. 1975, 1976, 1978).

Materials and Methods

Animals

Female B6C3F₁ mice (Glaxo Wellcome Research & Development), 12–14 weeks old, were housed in groups of five or six, bedded on wood shavings and given diet (Rat and Mouse No. 1, SDS Ltd., Witham, Essex) and drinking water ad libitum. An environmental temperature of 22 °C ± 1 °C was maintained, with a relative humidity of 45%–70% and a 12:12 h light:dark cycle (lights on at 06.00 h). Animals were acclimatised for at least 5 days before the start of the experiment and were observed daily for signs of ill-health, but more frequently after each BU dose.

Dosing

Busulphan. BU (Sigma Chemical Co. Ltd, Poole, Dorset) was dissolved in acetone at a concentration of 16.7 mg/ml. The BU solution was administered by intraperitoneal injection after dilution with distilled water (Water for Irrigations, BP) to give a constant dose volume of 10 ml/kg bodyweight. Control mice were given acetone with distilled water at the same dose volume.

Chloramphenicol. Chloramphenicol succinate (CAPS), (Sigma) was dissolved in distilled water and administered in the drinking water at 4.0 mg/ml.

Experimental Design

A total of 336 mice were randomly assigned to six groups of 36 (groups 1 to 6) and six groups of 20 (groups 7 to 12). Animals in groups 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12 were dosed on four

fortnightly occasions (days 1, 16, 30, and 43) with BU at 0 (controls), 10, 18, 25, 33 and 40 mg/kg, respectively. Six weeks after the fourth dose of BU, on day 85, animals in groups 1 to 6 were given CAPS in the drinking water ad libitum whilst those in groups 7 to 12 were given drinking water without CAPS.

Haematological Measurements

Animals were killed by CO₂ overdose and 0.5 ml blood samples were taken from the posterior vena cava and anticoagulated with K₂EDTA. Samples were taken on days 1, 49 (6 days after the last BU dose), 85 (the first day of CAPS administration), 140, 253, 384, and on day 485 or 497. In addition, on days 177 and 210, 0.2 ml blood samples were taken by caudal venepuncture. With the exception of greater variability of platelet counts, the data from these caudal vein bleeds were comparable to samples taken from the posterior vena cava. For logistical reasons, groups 1 to 6 were bled for the last time on day 485, and groups 7 to 12 on day 497. Blood was analysed using a Bayer H*1 haematology analyser with mouse-specific software (Bayer Ltd, Newbury, Berks) and a Sysmex R-1000 reticulocyte analyser (Sysmex UK Ltd, Milton Keynes, Bucks). Bone marrow cellularity was assessed on days 1, 49, 85 and 384 by flushing the contents of one femur into 10 ml of phosphate-buffered saline and, after thorough mixing, obtaining the nucleated cell count from the basophil (leucocyte) channel of the H*1. Smears made from the opposite femur were stained with May–Grünwald–Giemsa stain and myeloid, erythroid and lymphoid cells enumerated in differential counts of 200 cells. Also on days 1, 49, 85 and 384, cellularity of spleens was measured using the H*1, in suspensions prepared by scissor-mincing and dispersion by cavitation with a 50 ml syringe. Samples of blood, bone marrow and spleen were obtained, where possible, from all animals that died or were killed due to ill-health. In these cases, spleen imprints were made by touching the cut surface of the organ on a microscope slide which was subsequently stained with May–Grünwald–Giemsa stain.

Statistical Analysis

Student's *t* test, using RS/1 software (BBN, Cambridge, MA), was used to compare treated and control groups at the same timepoint. Two-way analysis of variance was used to compare data on days 485 and 497.

Results

Scheduled Bleeds

Statistical analysis of the results at each BU dose level in groups 1 to 6 (those receiving CAPS in drinking water) against groups 7 to 12 (those given water without

Table 1. Total femoral nucleated cells and myeloid, erythroid, and lymphoid cell counts at scheduled kills in B6C3F₁ mice given BU on days 1, 16, 30 and 43. Figures are mean counts $\times 10^7$ (SD), $n=5$

Busulphan dose (mg/kg)	Day of experiment				
	1	49	85	384	
Total cells	0	1.97 (0.39)	1.88 (0.52)	2.52 (0.93)	3.56 (0.59)
	10		1.89 (0.12)	1.90 (0.63)	3.32 (0.36)
	18		2.01 (0.48)	2.23 (0.74)	3.68 (0.83)
	25		2.15 (0.23)	1.56 (0.39)	3.72 (0.99)
	33		2.30 (0.60)	2.50 (0.44)	3.20 (0.59)
	40		1.38 (0.69)	2.16 (0.92)	3.46 (0.44)
Myeloid	0	0.82 (0.18)	0.78 (0.19)	0.93 (0.32)	1.23 (0.20)
	10		0.63 (0.19)	0.74 (0.24)	1.19 (0.17)
	18		0.56 (0.28)	0.91 (0.40)	1.30 (0.33)
	25		0.63 (0.11)	0.56 (0.11)	1.40 (0.28)
	33		0.73 (0.33)	1.02 (0.18)	1.31 (0.18)
	40		0.66 (0.50)	0.89 (0.34)	1.66 (0.60)
Erythroid	0	0.56 (0.13)	0.58 (0.25)	0.73 (0.31)	1.23 (0.30)
	10		0.56 (0.15)	0.61 (0.24)	1.11 (0.17)
	18		0.71 (0.24)	0.64 (0.24)	1.27 (0.25)
	25		0.68 (0.03)	0.48 (0.14)	1.30 (0.45)
	33		0.75 (0.23)	0.86 (0.32)	0.84 (0.25)
	40		0.45 (0.21)	0.66 (0.25)	0.96 (0.27)
Lymphoid	0	0.54 (0.12)	0.47 (0.19)	0.77 (0.39)	1.03 (0.24)
	10		0.69 (0.17)	0.48 (0.17)	0.95 (0.21)
	18		0.70 (0.16)	0.62 (0.15)	1.03 (0.24)
	25		0.78 (0.22)	0.50 (0.21)	0.98 (0.32)
	33		0.79 (0.09)*	0.53 (0.21)	0.91 (0.25)
	40		0.24 (0.06)	0.53 (0.33)	0.75 (0.27)

*Significantly different from controls, $p<0.05$.

CAPS), revealed no effects on haematological parameters that could be ascribed to the administration of CAPS. Therefore, the data discussed below are pooled from each BU dose level (ie groups 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12).

Bone Marrow. Bone marrow cellularity, assessed by total nucleated cell counts on days 1, 49, 85 and 384, showed no significant differences between BU-dosed and control animals (Table 1). There was an age-related increase in total cellularity, but the relative proportions of myeloid, erythroid and lymphoid precursors remained relatively constant. Absolute numbers of myeloid cells showed no BU-related changes and, similarly, absolute numbers of erythroid cells from BU-treated mice showed no significant variations from control values. However, lymphoid cell counts in animals dosed with 40 mg BU/kg were depressed to 51% of control levels at day 49 and they remained low until day 384 when the count was 73% of the control value. However, these decreases did not achieve statistical significance. At day 49, in the other BU-dosed groups of mice, lymphoid counts were elevated above control values. For example, in animals receiving 33 mg BU/kg, the counts were 167% of the

control figure ($p<0.05$). At day 85, lymphoid cell counts from BU-treated mice were reduced to between 62% and 81% of the control value, but recovered to between 87% and 99% of the controls in the 10–33 mg BU/kg groups at day 384. Megakaryocyte numbers were not affected by BU treatment. There were no apparent morphological changes in any cell line in BU-dosed mice.

Spleen Counts. Splenic nucleated cell counts, showed a clear dose-dependent depression on day 49 (Table 2), with values for animals receiving 40 mg BU/kg being 73% of controls ($p<0.001$). At day 85, counts for treated mice at all dose levels were similar, ranging between 67% and 76% of controls. By day 384, counts in animals receiving 10, 18 and 25 mg BU/kg had recovered to near normal values, whereas in the 33 and 40 mg BU/kg groups, counts were 78% and 80% of controls, respectively. Absolute spleen weights and splenic nucleated cell counts assessed on day 384 correlated closely (Fig. 1).

Erythrocytes. Six days after the final administration of BU, erythrocyte (RBC) counts (Fig. 2), haemoglobin (Hb) and haematocrit (HCT) were reduced in animals receiving 40 mg BU/kg; however, these reductions were

Table 2. Total splenic nucleated cell counts at scheduled kills in B6C3F₁ mice given BU on days 1, 16, 30 and 43. Figures are mean counts $\times 10^7$ (SD), $n=5$

Busulphan dose (mg/kg)	Day of experiment			
	1	49	85	384
0	16.35 (1.83)	15.81 (1.36)	17.96 (2.79)	29.08 (4.32)
10		14.68 (2.94)	13.68 (1.43)*	27.40 (5.15)
18		14.26 (1.77)	13.54 (1.42)*	32.30 (9.01)
25		12.82 (1.56)**	11.96 (2.79)**	30.93 (9.27)
33		12.69 (2.29)**	12.06 (1.63)**	22.75 (3.21)*
40		11.57 (0.99)***	12.79 (1.53)**	23.22 (5.28)

*Significant difference from controls: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

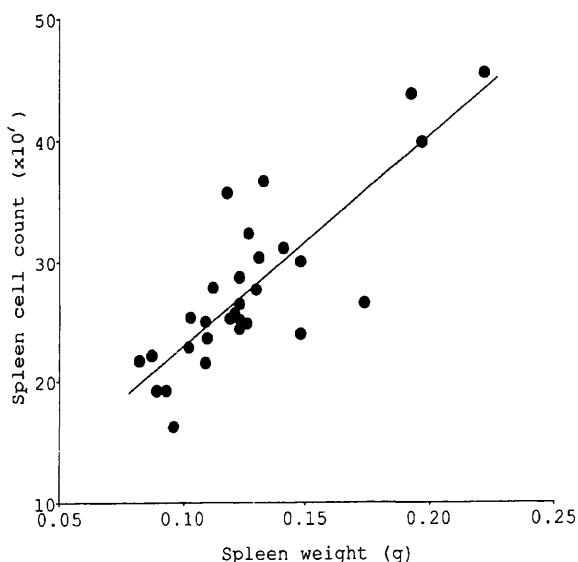


Fig. 1. Relationship between splenic nucleated cell count and spleen weight at day 384. Individual data are presented from control and BU-treated groups (10–40 mg/kg). $r=0.83$.

not statistically significant. The erythrocyte counts had recovered in the 40 mg BU/kg group by day 85. However, mean RBC values for all BU-treated groups remained lower, but not significantly lower, than controls from day 85 to the end of the experiment, with the exception of the 25 mg BU/kg group on day 140, and the 10 mg BU/kg group on day 253. After day 253, RBC counts in control and all BU-treated groups decreased slightly. There were no significant dose-related changes in any of the erythrocyte indices, i.e. mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, red cell distribution width, or haemoglobin distribution width.

Reticulocyte counts were not significantly affected by BU treatment at any dose level on any occasion. Counts in all groups tended to be the reverse of the erythrocyte counts, falling steadily until day 253 and rising slightly thereafter. Reticulocyte fluorescence ratios showed no BU treatment-related effects.

Leucocytes. With the exception of day 85, the total leucocyte count was reduced at 40 mg BU/kg to 50% to 80% of control values on all occasions after BU dosing. This was due to a reduction of all leucocyte types and achieved significance on days 210 ($p<0.001$), 253 ($p<0.001$), 384 ($p<0.05$) and 485/497 ($p<0.01$). At 10 and 18 mg BU/kg, a sustained increase in leucocyte count was seen; however, this increase was only statistically significant in the 10 mg BU/kg group on day 253 ($p<0.02$).

For the lymphocyte count, a marked depressive effect was seen in the 40 mg BU/kg group with the value reduced to between 51% to 66% of the control level on all occasions, except at day 85. These reductions were statistically significant on each occasion, with the exception of days 49 and 140, as follows: day 177, $p<0.01$; day 210, $p<0.001$; day 253, $p<0.001$; day 384, $p<0.05$; day 485/497, $p<0.01$. A dose-dependent trend was observed for the 33 mg/kg dose level, with statistically significant reductions seen on days 177 ($p<0.05$), 210 ($p<0.001$), 253 ($p<0.01$) and 485/497 ($p<0.05$). In contrast, a trend for elevation of the lymphocyte count was seen at 10 mg BU/kg, and to a lesser degree at 18 mg/kg. Statistical significance was achieved in the 10 mg BU/kg group on days 210 ($p<0.05$) and 253 ($p<0.05$), and in the 18 mg BU/kg group on day 85 ($p<0.01$). In addition to these changes in the lymphocyte count, the large unstained cell (LUC) count was depressed on all occasions in the 40 and 33 mg BU/kg groups, with no upward trend at day 85. The differences from control values were significant at the $p<0.01$ level in the 40 mg BU/kg group on days 85, 140, 210, 253, 384 and 485/497, and in the 33 mg BU/kg group on days 253, 384 and 485/497. However, unlike the lymphocyte counts, LUC values were not elevated at lower dose levels.

Neutrophils were depressed to between 67% and 93% of control values on day 49 in the various BU treatment groups. However, these reductions did not achieve statistical significance; thereafter there were no treatment-related effects, except on day 485/497 in animals receiving 33 and 40 mg BU/kg which showed group means of 66% and 63% of controls, respectively; the reduction was statistically significant at 40 mg/kg

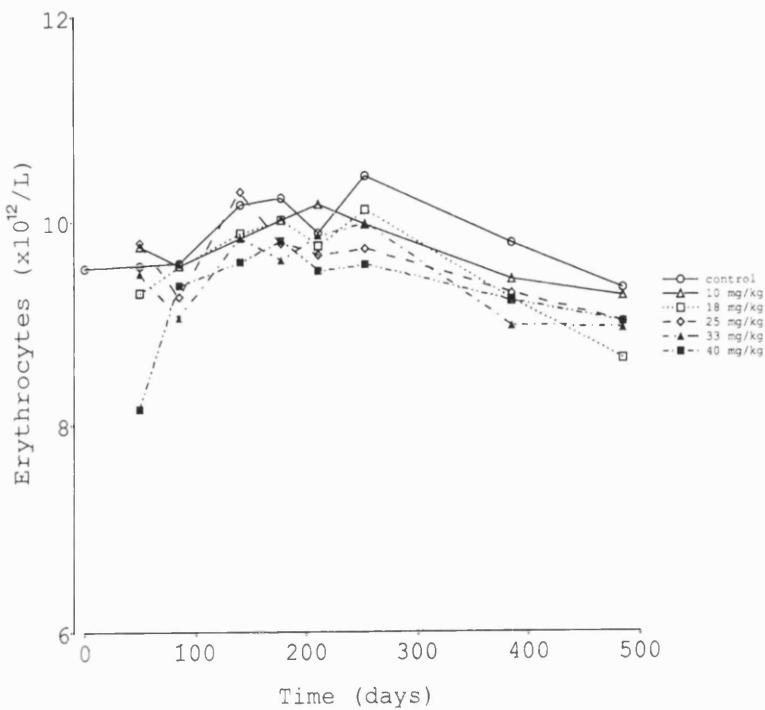


Fig. 2. Mean erythrocyte counts at scheduled bleeds in B6C3F₁ mice given BU (see key for dosage). $n=5$ (days 1, 49, 85 and 384), on all other occasions, $n=10$, except on day 485/497 where $n=9$ in control and 10 mg BU/kg groups.

($p<0.05$). Neutrophil myeloperoxidase content (MPXI, myeloperoxidase index), showed no alteration consistent with BU treatment.

Monocyte counts in all BU-dosed groups were depressed to between 48% and 89% of control values on day 49 (Fig. 3), with group mean counts of those animals receiving 18 and 33 mg BU/kg significantly different from the control mean ($p<0.01$ and $p<0.05$, respectively). Counts in all groups recovered to values

closer to control levels (between 77% and 124% of controls) between days 85 and 177, and were not significantly different from the control mean. Counts of monocytes in animals receiving 10, 18 and 25 mg BU/kg remained between 72% and 121% of control values from day 210 to days 485/497, and again, these counts were not significantly different from the controls. However, in mice receiving 33 and 40 mg BU/kg, group mean counts remained depressed from day 210 to day 485/497 to

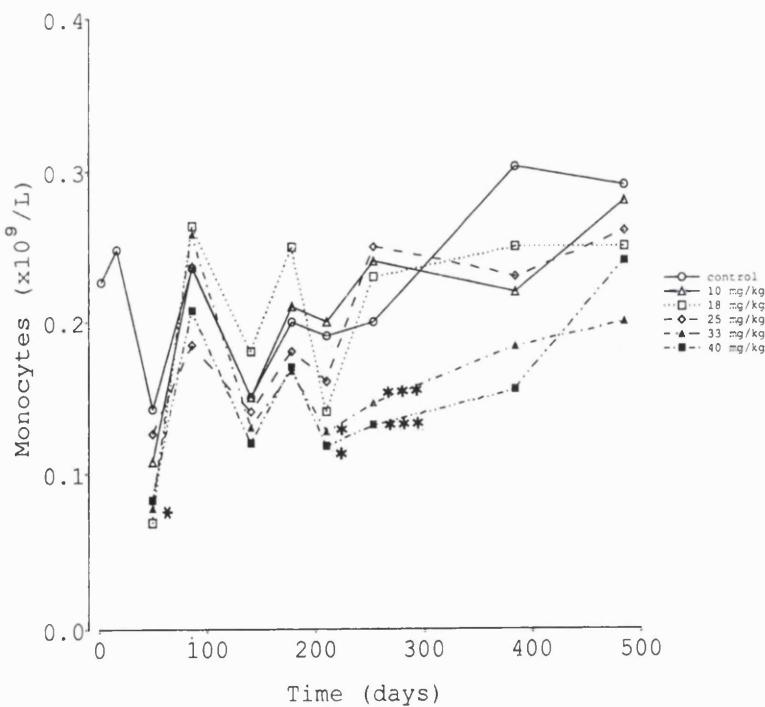


Fig. 3. Mean monocyte counts at scheduled bleeds in B6C3F₁ mice given BU (see key for dosage). For details of n , see Fig. 2. Significantly different from controls, $*p<0.05$, $***p<0.001$.

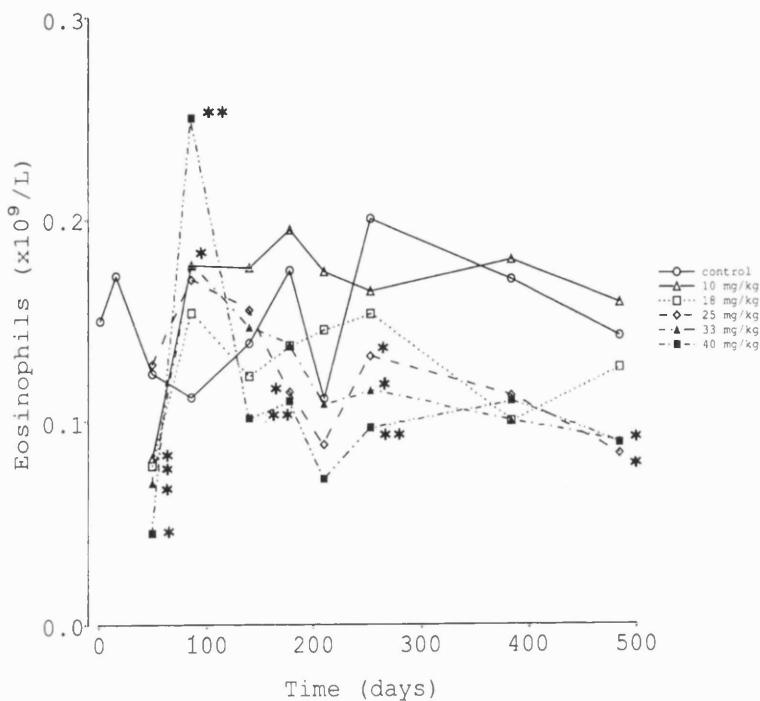


Fig. 4. Mean eosinophil counts at scheduled bleeds in B6C3F₁ mice given BU (see key for dosage). For details of *n*, see Fig. 2. Significantly different from controls, **p*<0.05, ***p*<0.01.

between 51% and 82% of the control group mean. On day 210 and day 253, the means of both these dose groups were significantly different from controls (*p*<0.05, day 210; *p*<0.01, day 253).

Counts of eosinophils displayed similar patterns of response to those of the monocytes. Counts in all groups except at 25 mg BU/kg were statistically significantly depressed (*p*<0.05) to between 36% and 60% of control levels at day 49 (Fig. 4). Counts then rose above the control values in all treated groups on day 85, with the 40 mg BU/kg group mean reaching 223% of the control value (*p*<0.01). For the rest of the study, counts in animals receiving 10 and 18 mg/kg were close to control values. Counts in the animals receiving 25, 33 and 40 mg BU/kg fell progressively from day 140 to between 48% and 66% of control values on days 253, 384 and 485/497; in those animals receiving 40 mg BU/kg, counts remained between 48% and 73% of controls from day 140 onwards. These counts were significantly different from the control mean in the 25 mg BU/kg group on days 177 and 485/497 (*p*<0.05 on each occasion); in the 33 mg BU/kg group on days 253 and 485/497 (*p*<0.05 on each occasion); and in the 40 mg BU/kg group on days 177 and 253 (*p*<0.01 on each occasion).

Basophil counts were consistently reduced from day 85 onwards in animals receiving 40 mg BU/kg to around 50% of control mean counts; these reductions did not achieve statistical significance.

Platelets. Platelet counts showed considerable variability on different occasions, with standard deviations for control group animals ranging between <40 and >400 $\times 10^9/l$ (coefficients of variation of <3.6% to >36% for a control count of $1100 \times 10^9/l$). This variability was

attributed to blood sampling difficulties, and was especially noticeable in the data on days 177 and 210; data for these occasions is not presented. Highly statistically significant reductions in platelet count were seen in all BU dose groups up to day 140 of the study. With the exception of animals receiving 10 mgBU/kg on day 49, all reductions were significant with *p* values of at least <0.01 (Fig. 5). These reductions were not clearly dose-related, although a distinct trend was discernible on most occasions until day 140. Counts in animals receiving 40 mg BU/kg were reduced on all occasions to between 37% and 87% of control values; on day 253 this reduction was still statistically significant (*p*<0.01).

Intercurrent Deaths

Mortality. As most mice showed significant dose-related haematological changes only at the highest BU dose levels, a number of mice were killed when their condition began to deteriorate rapidly and some others were found dead ('intervent deaths' or ICDs). Most ICDs occurred in the group receiving 40 mg BU/kg and by day 497, survival in that group was 41% (Fig. 6). Survival at day 497 was 73% in the 33 mg/kg group, and 93% in the 25 mg/kg group. Survival in the controls, and animals receiving 10 and 18 mg BU/kg was similar, ranging between 95% and 97% at day 497. Deaths occurred early in the study (in the first 200 days) in the 40 and 33 mg BU/kg groups but not until after this time in mice receiving 25 mg BU/kg. Controls and mice receiving 10 and 18 mg BU/kg showed no mortality until nearly one year after the start of the study.

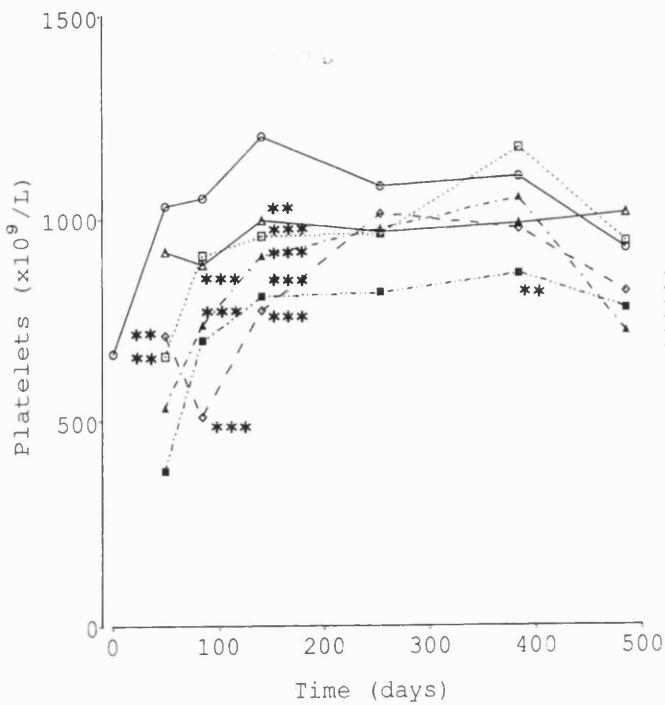


Fig. 5. Mean platelet counts at scheduled bleeds in B6C3F₁ mice given BU (see key for dosage). For details of *n*, see Fig. 2. Significantly different from controls, ***p*<0.01, ****p*<0.001.

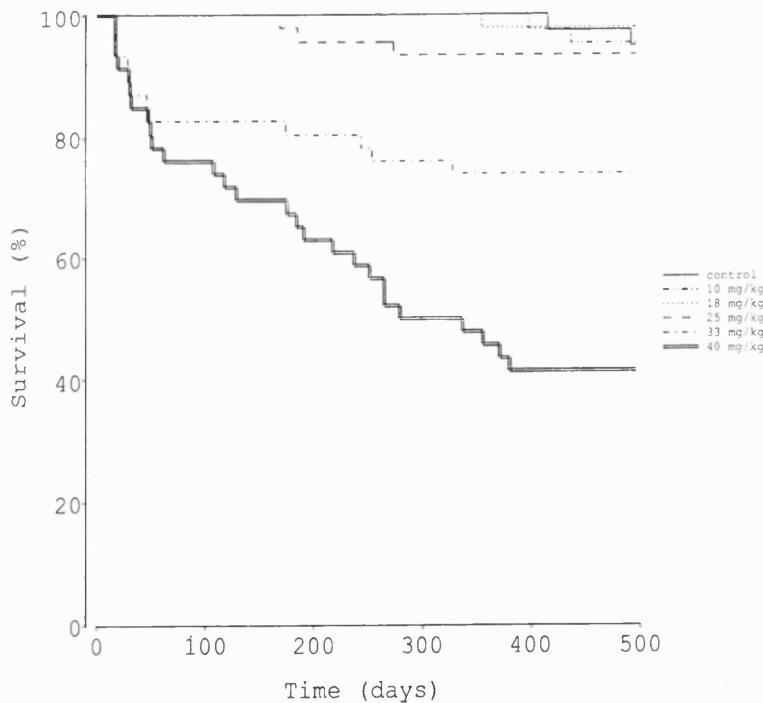


Fig. 6. Survival in B6C3F₁ mice given BU (see key for dosage) on days 1, 16, 30 and 43. Survival is expressed as the percentage survivors of the number of animals in each BU-treated group at day 1 of the experiment.

Bone Marrow. Marrow samples from all but two of the 25 animals dying before day 175 were moderately or markedly aplastic, with nucleated cell counts less than 1.0×10^7 and in two cases less than 0.1×10^7 (Fig. 7). For comparison, the range (expressed as 2 of the SD mean) of nucleated cell counts for control mice on day 1 was $1.19\text{--}2.75 \times 10^7$ and on day 384, 2.38 to 4.74×10^7 . Where mice were found dead and it was not possible to obtain femoral samples for counting, smears were made.

Smears from all the ICDs prior to day 175 (with one exception) revealed depletion of megakaryocytes, myeloid and erythroid precursors and in some cases the only cells present were lymphoid cells and reticuloendothelial macrophages. After day 175, cellularity, as assessed by nucleated cell count in ICD animals, was within, or close to, normal values but, in the majority of cases, normal haematopoietic cells were replaced by lymphoma cells.

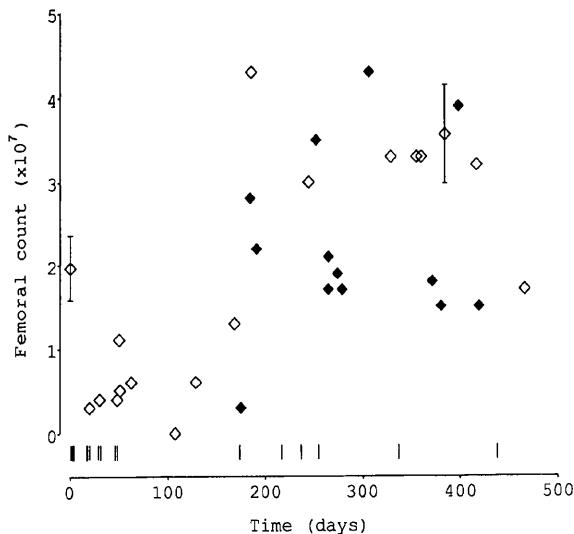


Fig. 7. Total femoral nucleated cell counts in intercurrent death B6C3F₁ mice given BU on days 1, 16, 30 and 43 (from 10 to 40 mg/kg). ♦ indicates animals in which there was infiltration of femoral marrow by lymphoma cells; ◇ no infiltration. Days on which animals died but from which no data were obtained, are shown as vertical 'ticks' above the x axis. Symbols with error bars show the mean control values (\pm SD) on days 1 and 384.

Spleen. Spleen cellularity was depressed in animals dying before day 175 (Fig. 8). Some ICD animals dying after this time had normal counts but in others the numbers were moderately or markedly increased. In one case (not shown in Fig. 8) on day 254, the count was 1660×10^7 ; the control range (expressed as 2SD of the mean) on day 384 was $20.44-37.40 \times 10^7$. In spleen

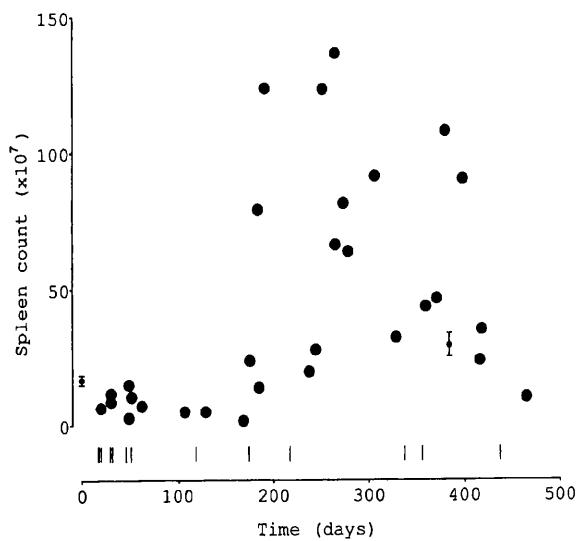


Fig. 8. Total splenic nucleated cell counts in intercurrent death B6C3F₁ mice given BU on days 1, 16, 30 and 43 (from 10 to 40 mg/kg). Days on which animals died but from which no data were obtained, are shown as vertical 'ticks' above the x axis. Symbols with error bars show the mean control values (\pm SD) on days 1 and 384.

imprints from ICD cases after day 175, the majority of lymphoid cells appeared neoplastic with morphological characteristics similar to the cells infiltrating the marrow.

Erythrocytes. In animals dying before day 175, both erythrocyte and reticulocyte counts were markedly depressed. Where counts could not be performed because of insufficient sample volume or where animals were found dead, reticulocyte percentage was estimated from the assessment of polychromasia in stained films. After day 175, reticulocytes were variously depressed or close to control values (Fig. 9). However, about one-third of reticulocyte counts greatly exceeded those of the control. In these cases although bone marrow smears showed infiltration by lymphoma cells, moderate to marked extramedullary haematopoiesis, especially erythropoiesis, was apparent in spleen impressions.

Leucocytes. Neutrophil counts were below control values in all but one of the ICD mice sampled before day 175. Counts were normal or increased thereafter (Fig. 10). Lymphocytes in all ICD mice sampled before day 175 were below the control range; thereafter, the counts were inconsistently varied. Lymphocyte counts and spleen cellularity were generally closely correlated (Fig. 11). However, in the individual case mentioned above (Section on Spleen) the spleen count was 1660×10^7 whereas the lymphocyte count was $2.32 \times 10^9/l$ which was below the control range (expressed as 2SD of the mean) for day 253 ($2.79-7.15 \times 10^9/l$). The proportion of leucocytes classified by the H*1 analyser as LUC was elevated in the majority of ICD cases, both before and after day 175. The LUC counts correlated well with spleen cellularity. In more than half the samples with

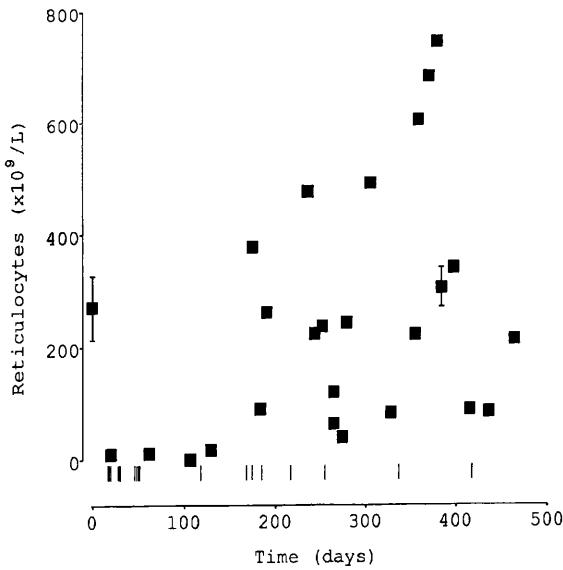


Fig. 9. Reticulocyte counts in intercurrent death B6C3F₁ mice given BU on days 1, 16, 30 and 43 (from 10 to 40 mg/kg). Days on which animals died but from which no data were obtained, are shown as vertical 'ticks' above the x axis. Symbols with error bars show the mean control values (\pm SD) on days 1 and 384.

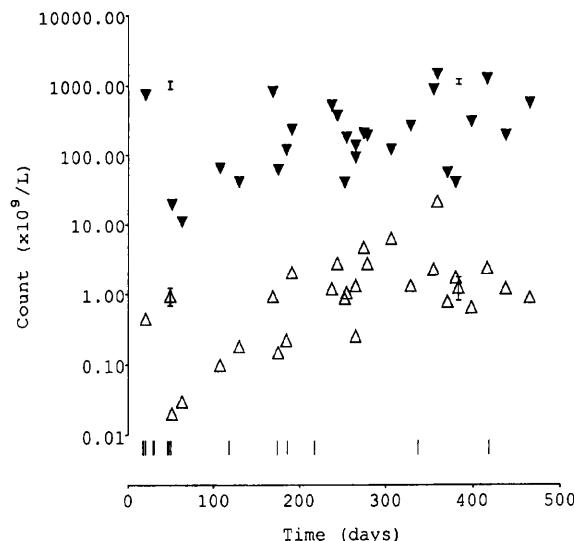


Fig. 10. Neutrophil (Δ) and platelet (∇) counts in intercurrent death B6C3F₁ mice given BU on days 1, 16, 30 and 43 (from 10 to 40 mg/kg). Days on which animals died but from which no data were obtained are shown as vertical 'ticks' on the x axis. Symbols with error bars show the mean control values (\pm SD) on days 49 and 384.

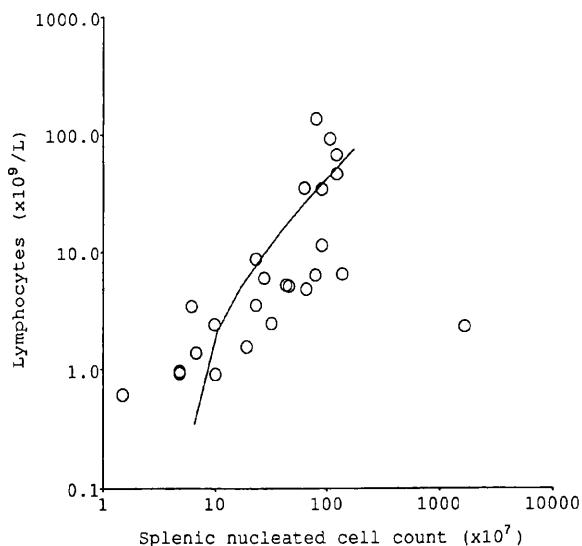


Fig. 11. Relationship between total splenic nucleated cell counts and lymphocyte counts in intercurrent death animals. The outlier is the case sampled on day 254, giving a spleen count of 1660×10^7 and a lymphocyte count of 2.32×10^9 /L; the line of best fit excludes this individual.

elevated LUCs, the percentage of LUCs (of the total leucocyte count) was greater than 50%. The morphology of these LUCs was consistent with that of the lymphoma cells infiltrating the marrow and spleen. Cytochemical staining of these cells for acid phosphatase and α -naphthyl acetate esterase gave localised reactions in the majority of cells, suggesting a T cell lineage.

Platelets. The pattern of changes seen in the platelet counts of the ICD mice paralleled the sequence of the neutrophil response with the platelet counts being depressed before day 175 and approaching normal after this time (Fig. 10).

Discussion

In previous work to establish the maximum tolerated intraperitoneal dose of BU in female B6C3F₁ mice (Andrews et al. 1992, 1993a,b), transient reductions of leucocyte, erythrocyte and platelet counts were seen after single doses of 35 and 45 mg BU/kg. Femoral and splenic cellularity was reduced immediately after dosing but was restored within a few days with erythropoiesis exhibiting a 'rebound' response whereas leucocyte and platelet production was impaired for a longer period. After dosing at 10–40 mg BU/kg on four occasions at fortnightly intervals, reductions in erythrocyte, leucocyte and platelet counts were depressed at six weeks after treatment (Andrews et al. 1993b).

In the present work, where BU was administered intraperitoneally on four occasions at fortnightly intervals, we have observed a sustained depression of leucopoiesis and thrombopoiesis in animals receiving 33 and 40 mg BU/kg. Although the persistent depressive effect was shown in all leucocyte types, lymphocytes showed the greatest sensitivity. As reported earlier (Andrews et al. 1997), the effect was clearly demonstrable in circulating B cells; a reduction in lymphoid numbers was also seen in the bone marrow and spleen of animals given 33 and 40 mg BU/kg. Unexpectedly, it was also found that in animals receiving the lowest dose of BU (10 mg/kg) there was a persistent, mild, elevation of lymphocyte counts, but not in the counts of LUCs.

In animals surviving until the end of the present study (day 485/497), there was no effect on erythropoiesis at any BU dose level: peripheral counts of erythrocyte and reticulocytes, including HFR reticulocytes, remained normal and there was no reduction in erythroid precursors in the bone marrow. It was also found that dosing mice with CAPS at 4 mg/ml in the drinking water produced no additional toxicity. Water consumption data showed that the average consumption of CAPS throughout the experiment was of the order of 170–370 mg/kg/day. Recent studies have shown that in the female CD-1 mouse, dose levels of CAPS administered daily by gavage in excess of 1400–1700 mg/kg for 7–10 days are required to induce an acute reversible anaemia (Yallop et al. 1998).

Our findings with BU and CAPS support the results of other workers who have attempted to repeat Morley's studies. Pazdernik and Corbett (1980), using Morley's BU dosing regime in BDF₁ mice, gave CAPS in drinking water at 5 mg/ml, starting two weeks after the last BU dose, for six weeks. CAPS caused no enhancement of the BU-induced reduction of femoral cellularity. Pazdernik and Corbett did not report any late deaths due to aplasia in their mice. However, their experimental evaluation of

haematopoiesis and the start of CAPS administration was at a much earlier time than that described by Morley. In an investigation by Robin et al. (1981), the appearance of lymphoma, rather than aplasia, in BU + CAP-treated mice, led these workers to modify their experimental objectives which then became focussed on the characterisation of the induced lymphoma (Bhoopalam et al. 1986).

Animals in the present study which received the highest doses of BU (33 and 40 mg/kg) succumbed to the drug in one of two ways. First, up to day 175 (19 weeks after the cessation of BU dosing), animals died as a result of marrow aplasia and profoundly depressed peripheral counts of all cell types. It is not clear whether these individuals developed late onset aplasia, since it was not feasible to perform serial blood counts. Some of the early deaths are attributed to the acute effects of BU. In Morley and Blake's (1974a) experimental protocol, which used the BU dosing regime adopted here, animals were included in the study if they survived longer than six weeks after the cessation of BU dosing. Using this criterion, deaths occurring after day 85 in the present study are considered to be the result of a late-onset aplastic effect. The second way in which animals succumbed, which was evident after day 175, was where lymphoma supervened and in these animals, other aspects of haematopoiesis were relatively normal.

In the present investigation, the purpose of the experimental design was to examine the work of Morley and his co-workers (Morley and Blake 1974a, b; Morley et al. 1975, 1976) and establish a model of aplastic anaemia in the B6C3F₁ mouse. However, although we have demonstrated some effects of BU similar to those seen by these workers in the BALB/c and Swiss mouse, we have encountered unexpected differences. The B6C3F₁ mouse is able to tolerate a higher dose level of BU than some other strains (Andrews et al. 1993b). Strain difference may also account for a lack of susceptibility of the B6C3F₁ mouse to CAP (Miller et al. 1978; Festing 1987). It is possible that increased clearance of CAP or the generation of non-myelotoxic metabolites in the B6C3F₁ mouse, causes much lower exposure of target haematopoietic precursors in this strain than in the BALB/c or Swiss mouse.

Morley and Blake (1974a) noted persistently depressed bone marrow erythroid numbers and lowered haematocrit in mice up to 240 days after receiving a total of 70 mg BU/kg. Depressed marrow erythropoiesis was only seen in early ICDs in the present study and was not a feature of the haematology of the surviving animals. A proportion of the mice in Morley and Blake's (1974a) report also developed leukaemia/lymphosarcoma. It would therefore appear that this neoplasm developed in some mice in parallel with the aplasia that was developing concurrently in other animals. This contrasts with the results in the present study where some mice developed aplasia in the early stages of the experiment whereas lymphoma was seen in other animals at a later stage.

The development of lymphoma in BU-treated mice has been reported by other workers. Administration of 20 mg BU/kg induced thymic lymphoma in 12% of RF/Up mice at a median time of 10 months; marrow aplasia was not reported (Upton et al. 1961, Karnofsky 1967). Robin et al. (1981) found that administration of CAP increased the incidence of lymphoma to 35% in mice 6 months after being given 20 mg BU/kg on four fortnightly occasions; again, aplasia was not reported. Hunstein (1965) reported that rats receiving 0.3 or 0.5 mg BU weekly became aplastic, but at 5–6 months developed leukaemia (monotypic hyperplasia).

BU has been shown to induce marrow hypocellularity in mice after a single dose ranging between 5 to 100 mg/kg (Udupa et al. 1972; Josvasen and Boyum 1973; Boggs and Boggs 1980; Jelkmann and Bauer 1980). The marrow and peripheral blood counts reported by these authors are similar to those described in our earlier work (Andrews et al. 1993b). Using the regime of Morley and Blake (1974a) with BU in Swiss mice, other workers have successfully induced bone marrow hypocellularity with a reduction of stem cells (CFU-S) and/or myeloid progenitors (CFU-C or CFU-GM) (Iderha et al. 1984; Boyd et al. 1986; Halka et al. 1987). Similar findings have been reported with BU administered to CF₁ mice (Wathen et al. 1982), in C3H hej mice given a total of 20 mg BU/kg (Fitchen and Cline, 1980) and in C57BL/6 (H-2^b) mice receiving 136 mg BU/kg over four days (Down et al. 1989). However, A/J mice treated with BU using Morley's regime developed bone marrow hypoplasia with profoundly reduced CFU-C numbers at 40 weeks after dosing but, in contrast, displayed compensatory splenic erythropoiesis (McManus and Weiss 1984). On the other hand, Botnik et al. (1979) gave C3H/HeJ mice 6 weekly doses of BU totalling 2.04 mg and whilst noting a decreased potential of stem cells to proliferate, demonstrated no aplasia in the treated animals after 80 weeks.

Thus, the fundamental property of BU to induce transient hypocellularity in the mouse, as first described by Elson (1955) in the rat, is well established and reproducible. Many authors have gone on to confirm Morley's hypothesis that BU damages stem cells (Morley et al. 1975) rather than committed erythroid cells (Jelkmann and Bauer 1980), or committed granulocytic cells (Fitchen and Cline 1980). Evidence for damage to the marrow microenvironment by BU has also been demonstrated (Wathen et al. 1982; McManus and Weiss 1984). However, the phenotypic manifestation of stem cell damage, that is susceptibility to CAP-induced late-onset marrow aplasia with profoundly depressed peripheral cell counts, would appear to be less reproducible. Equally, the development of lymphoma is by no means consistent in the literature reports.

Similar inconsistencies exist in the literature on murine hypoplasia and hyperplasia induced by benzene (Farris et al. 1993), a chemical long associated with AA, myelodysplasia and myeloid leukaemia in humans. Both BU and benzene damage stem cells, but whereas BU is a potent alkylator (Hall and Tilby 1992), benzene

metabolites cause chromosomal damage (Irons and Stillman, 1996) but in neither case is the pathogenesis of lymphoma clear-cut. A number of processes are thought to be involved including activation of endogenous retroviruses, altered cytokine responses and decreased p53-dependent apoptosis, leading to promotion of neoplastic proliferation (Irons et al. 1996). Some of these same processes may be implicated in the aetiology of AA in humans. However, successful therapy with antilymphocyte globulin argues for an immune-mediated component of AA in which the proliferation of an abnormal clone is prevented by cytotoxic T cells (Young 1996). Thus, although stem cell damage in animal models may be shown under various circumstances as chronic marrow hypoplasia (reduced self-replicating potential), or lymphoproliferative disorder (decreased apoptosis and neoplastic transformation), the development of late-onset marrow aplasia may depend on the immune status of the chosen rodent strain.

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The myelotoxicity of chloramphenicol: *in vitro* and *in vivo* studies: II: *in vivo* myelotoxicity in the B6C3F₁ mouse

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- 1 Chloramphenicol continues to be widely used in many parts of the world despite its known haematotoxicity. Until now, elucidation of the mechanisms involved and any attempt at amelioration of the toxic effects have been hampered by the lack of an animal model.
- 2 In this study neither acute nor chronic administration of chloramphenicol as its succinate ester in the drinking water produced anaemia in mice as assessed by changes in peripheral blood parameters.
- 3 Chloramphenicol could not be detected in the bone marrow when the antibiotic was administered either in the drinking water or by gavage, although it was detected in the serum.
- 4 In marrow taken from mice after chloramphenicol succinate administration and cultured *in vitro*, depression of the differentiation of immature committed erythroid progenitors occurred 15 min after administration of the antibiotic by gavage. However, recovery

was beginning to occur at 48 h after administration of chloramphenicol succinate at 50 and 200 mg/kg and this was then followed by an 'overshoot' response at the higher dose. A toxic effect was therefore achieved in the bone marrow but this was probably masked in the peripheral blood by enhanced proliferation.

- 5 Morphological evidence of apoptosis was seen in erythroid and myeloid precursors in mice treated with 200 mg/kg.
- 6 The data suggest that the effect of chloramphenicol was at the differentiation stage of the committed marrow progenitor cells rather than at the replication stage of the stem cells and therefore this response appears to mimic the reversible bone marrow depression seen in the treated patient.

Keywords: chloramphenicol; myelotoxicity; mouse; peripheral blood; apoptosis

Introduction

Despite its known haematotoxicity,^{1,2} chloramphenicol (CAP) is still widely used in many parts of the developing world for the treatment of meningitis³ and typhoid fever.⁴ It commonly causes a reversible, dose dependent depression of the bone marrow which leads to anaemia accompanied by thrombocytopenia and leucopenia⁵⁻⁸ and, in rare cases, an idiosyncratic and often fatal aplastic anaemia (AA).⁹⁻¹¹ The elucidation of the toxic mechanisms involved in these processes has been hampered by the lack of adequate models. There is evidence that CAP induces apoptosis in haematopoietic and other dividing cells *in vitro* and that this effect can be ameliorated by co-culture with antioxidants.¹² These observations suggest that treatment regimens may be devised which could ameliorate clinical manifestations of CAP toxicity, co-administration with an antioxidant vitamin for example. However there is no suitable animal model which reproduces CAP-induced haematotoxicity and in which the hypothesis could be tested.

Morley and co-workers¹³⁻¹⁷ developed a model of aplastic marrow failure in female Swiss and BALB/c mice using busulphan and a range of other cytotoxic drugs including CAP. Morley, Trainor and Remos¹⁶ administered busulphan on four occasions over a six week period to BALB/c mice, followed by CAP, as its succinate ester (CAPS) in the drinking water at a concentration of 5 mg/ml, for 20 weeks. This model showed that the administration of CAPS in the drinking water to normal mice, and mice having residual marrow damage, caused a progressive fall in the numbers of pluripotent stem cells only in those animals bearing busulphan-damaged marrow. These data suggested some predisposition to the effects of CAP and this was put forward as an explanation for the apparently idiosyncratic occurrence of AA in man after CAP treatment.

In investigations using the model of residual marrow damage of Morley *et al*, where mice were pretreated with busulphan and CAP was then administered as CAPS in the drinking water, we have been unable to establish marrow aplasia based on changes observed in the peripheral blood.¹⁸⁻²⁰ We now report studies on the development of a

model of CAP haematotoxicity in normal female B6C3F₁ mice. We describe a series of experiments examining (a) the effects on peripheral blood parameters of CAP administered as CAPS in the drinking water; (b) concentrations of CAP and CAPS in the serum and bone marrow; (c) growth in culture of bone marrow cells removed from treated mice, and (d) morphological changes in bone marrow cells induced by CAP administration.

Materials and methods

Haematological studies

Animals Female B6C3F₁ mice (Glaxo Wellcome Research and Development), 12–14 or 26 weeks old, were housed in groups of four to six, on wood shavings, with diet (Rat and Mouse No. 1, SDS Ltd, Stepfield, Witham, Essex) and mains drinking water *ad libitum*. A temperature of 22±1°C was maintained, with a relative humidity of 45–70% and a light:dark cycle of 12:12 h (lights on at 06.00 h). Animals were acclimatised for at least 5 days before each experiment and were observed daily for signs of ill-health. Animals were weighed, and their water consumptions determined at appropriate times.

Administration of chloramphenicol Chloramphenicol as its succinate ester (CAPS, Sigma Chemical Company Ltd, Poole, Dorset) was prepared as a stock solution containing 100 mg CAPS/ml in sterile distilled water. The solution was diluted for administration to mice in the drinking water at concentrations of 1.0–6.0 mg CAPS/ml.

Haematological measurements Animals were killed by CO₂ overdose, 0.5 ml of blood removed from the posterior vena cava and anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacriston, Durham). The contents of the right femur were flushed into 2 ml phosphate buffered saline (PBS) to prepare a cell suspension, and a marrow smear prepared from the contents of the left femur. The spleen was removed, bisected, and a contact impression made with the cut face on a glass slide. The tissue was then scissor-minced and dispersed in PBS by cavitation in a 50 ml syringe to prepare a cell suspension.

Blood samples, bone marrow and spleen suspensions, were analyzed using a Technicon H*1 haematology analyzer (Bayer Diagnostics UK Ltd, Basingstoke, Hants) with mouse-specific software (Technicon, Swords, Co. Dublin, Eire). Measurements performed were: haemoglobin concentration (Hb), erythrocyte count (RBC), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), red cell dis-

tribution width (RDW), haemoglobin distribution width (HDW), percentage hypochromic erythrocytes (haemoglobin concentration <25 g/dl), percentage macrocytes (cell volume >70 fl), leucocyte count (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, large unstained cells (LUC), mean neutrophil peroxidase index (MPXI), platelet count, mean platelet volume (MPV), platelet distribution width (PDW). For the marrow and spleen cell suspensions, total nucleated cell counts were obtained from the basophil channel of the H*1. Reticulocyte analysis was performed using a Sysmex R-1000 instrument (Toa Medical Electronics Ltd, Milton Keynes, Bucks). Three equal divisions of the total number of reticulocytes gave the (percentage) low (L), medium (M) and high fluorescence ratios (HFR). Marrow smears and spleen impressions were stained with May-Grünwald-Giemsa stain, and differential counts performed by eye on 200 cells. Results are given as mean±s.d. Further details of the haematological measurements have been presented elsewhere.¹⁸

Trial designs *Experiment 1: Seven days study* - CAP was administered as CAPS in the drinking water to seven groups of 10, 12-week-old mice at concentrations of 0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg CAPS/ml from day one to day eight. On the eighth day the mice were killed and blood, marrow and spleen samples examined.

Experiment 2: 30 days study CAP was administered as CAPS in the drinking water to three groups of 30, 12 to 14-week-old mice at concentrations of 0, 2.5 and 4.0 mg CAPS/ml. At five, 10, 15, 20, 25 and 30 days, five mice per group were killed and blood, marrow and spleen samples prepared.

Experiment 3: 13 months study CAP was administered as CAPS in the drinking water to two groups of 30, 26-week-old mice at concentrations of 0 and 4.0 mg CAPS/ml. At 55, 92, 125, 167, 299 and 400 days, five mice per group were killed and blood, marrow and spleen samples examined.

Data analysis Student's *t* test was used to compare treated and control groups using a RS/1 software package (BBN Software, Cambridge, Massachusetts).

Assay of CAP and CAPS in serum and marrow

Animals Animals and their maintenance were as described above.

Administration of CAP Mice were dosed with CAP as CAPS, in the drinking water as described above, or by gavage. For administration by gavage, solutions of CAPS in sterile water were prepared and dosed at a constant volume of 10 ml/kg body weight.

Preparation of serum and marrow for the assay of CAP and CAPS *Sample preparation Serum* - Mice were killed by CO₂ overdose, blood removed as described above, added to Microtainers (Becton Dickinson, New Jersey, USA) and serum prepared. *Marrow* Mice were killed, marrow removed into 2 ml PBS as described above, and the cell suspension stored at -20°C.

Assay After thawing, samples (20 µl) of serum and disrupted bone marrow cells were analyzed for CAP and CAPS using the hplc method of Holt *et al.*²¹ The samples were extracted with ethyl acetate (1 ml) containing ethyltolylbarbituric acid (5 mg/L) as an internal standard. After vigorous mixing, the phases were separated by centrifugation (5 min, 2500 g) and the organic phase removed. An aliquote (900 µl) was evaporated under a stream of nitrogen and the residue redissolved in hplc mobile phase²¹ (200 µl) for analysis. The limit of detection of the assay was 1 mg/L for both CAP and CAPS.

Trial designs Experiment 1: 13 month study CAP was administered as CAPS in the drinking water at a concentration of 4.0 mg CAPS/ml to 20, 26-week-old mice for 400 days. These animals were part of a separate study and had been treated with busulphan over the period 14–20 weeks of age. It was assumed that this treatment would have no affect on the subsequent pharmacokinetics of CAP and CAPS, and the results were therefore included in the present study. At 400 days, the mice were killed by CO₂ overdose (at 11.00 h), serum prepared and assayed for CAP. A further four mice of the same age which had been given drinking water without CAPS were also included in this experiment as untreated controls.

Experiment 2: Circadian variation in CAP and CAPS concentration in serum and bone marrow - Forty-five mice (12-weeks-old) were divided into three groups of 16, 15, and 14 animals, and CAP administered as CAPS in the drinking water at CAPS concentrations of 0, 4.0 and 6.0 mg/ml respectively. In the 24 h period between days 15 and 16, three or four mice from each of the three dose regimens were killed at 19.00 h, 00.00 h, 06.00 h and 11.00 h, and serum and marrow samples prepared as described and assayed for CAP and CAPS. Measurements of water consumption were carried out daily from day one to 15, and in the 24 h between day 15 and 16 over the periods 11.00–19.00 h, 19.00–00.00 h, 00.00–06.00 h and 06.00–11.00 h.

Experiment 3: Administered by gavage Sixty mice were divided into three equal groups and CAP as CAPS in distilled water administered orally by gavage at concentrations of 25, 50 and 100 mg CAPS/kg body weight. Groups of five mice from each dose regimen were killed at 30 min, 1, 2 and 4 h, serum and marrow samples prepared as described and assayed for CAPS and CAP. A further

ten mice were given distilled water by gavage, five were killed at 30 min and five at 4 h as controls.

Data analysis Data are presented as means of groups (standard deviation).

Marrow cell culture

Animals Female B6C3F₁ mice (Glaxo Wellcome Research and Development), 12–14 weeks old, were housed in groups of 12, bedded on wood shavings and given diet (Rat and Mouse Standard Diet, B & K Universal Ltd, Grimston, Aldbrough, Hull), and drinking water from the mains supply *ad libitum*. A temperature of 20±1°C was maintained with a relative humidity of 45–65% and a light: dark cycle of 12 h:12 h (lights on at 07.00 h). Animals were acclimatized for at least seven days before the start of each experiment and were observed daily for signs of ill-health.

Administration of CAP Solutions of CAP as CAPS in distilled water were administered to mice by gavage, at a constant volume of 20 ml/kg body weight, to give doses of 0–200 mg CAPS/kg body weight.

Trial designs Experiment 1: Growth of marrow cells in vitro at 15 to 120 min following administration CAP was administered as CAPS to 40 mice at concentrations of 0, 25, 50, 100 and 200 mg CAPS/kg body weight. At 15, 30, 60 and 120 min after dosing, two mice from each dose regimen were killed, marrow removed and the cells cultured.

Experiment 2: Growth of marrow cells in vitro at 1 h to 28 days following administration CAP was administered as CAPS to 48 mice at concentrations of 0, 50 and 200 mg CAPS/kg body weight. At 1 and 2 h, and 1, 2, 4, 7, 21 and 28 days after dosing, two mice from each dose regimen were killed, marrow removed and the cells cultured.

Collection of marrow cells 1.0 ml Hypnorm (Janssen Animal Health) and 1.0 ml Hypnovel (Roche) were added to 4.0 ml sterile water and injected intraperitoneally at a dose of 6.7 ml/kg bodyweight. Under deep anaesthesia the cervical vertebral column was dislocated, the right or left femur removed and placed in sterile PBS. Using sterile techniques, the bone was cleaned of extraneous tissue and the epiphyses removed. With a 21 G needle on a 1 ml syringe, the marrow cells were aspirated into sterile PBS. The cells from two animals in each group were pooled and stored on ice for up to 3 h before being cultured.

Culture of marrow cells Bone marrow cells, harvested as described above, were resuspended in Iscove's modification of Dulbecco's medium (IMDM,

10 ml) containing fetal calf serum (FCS, 10%), L-glutamine (200 μ M, 100 μ l) and penicillin/streptomycin (10 000 units/10 000 μ g/ml, 100 μ l). Adherent cells were removed by depletion on plastic tissue culture dishes at 37°C overnight. The non-adherent cells were resuspended in fresh IMDM and counted. Cells (2×10^5 /ml per time point) were cultured in a semisolid medium of methyl cellulose (1% w/v in IMDM) containing FCS (30% v/v), 5637 conditioned medium (5% v/v), penicillin (100 units), streptomycin (100 μ g), β -mercaptoethanol (10 nM), bovine serum albumin (0.1% w/v), human erythropoietin (2 units; Eprex, Janssen-Cilag Ltd, High Wycombe, Bucks UK) and murine colony stimulating factor (10 ng; Sigma, Poole, Dorset) in a final volume of 1 ml. Quadruplicate samples (200 μ l) of the mixture were placed in 96 well tissue culture plates and incubated at 37°C. Colony forming units-erythroid (CFU-E) were counted at 7 days and burst forming units-erythroid (BFU-E) at 14 days.

Data analysis Data are presented as mean (standard deviation). Analysis of data was carried out using Student's *t*-test, assuming equal variance between groups.

Transmission electron microscopy of marrow cells Solutions of CAP as CAPS in distilled water were administered to two groups of four mice at concentrations of 0 and 200 mg CAPS/kg body weight. Marrow cells were removed from two animals in each dose regimen, as described above, at 2 h or 24 h post dosing. Non-adherent cells, prepared as described, were fixed as a loose mass in cacodylate buffered gluteraldehyde (3% in 0.1 M buffer, 30 min) followed by cacodylate buffer alone (30 min), and post-fixed in cacodylate buffered osmium tetroxide (1%, 30 min). After dehydration in industrial methylated spirit (IMS; 50%, 70%, 90%, 15 min each) and 100% IMS (30 min), ethanol (30 min) and propylene oxide (30 min), the cells were embedded in araldite. Ultrathin (50–60 nm) sections were cut and mounted on copper grids before staining with uranyl acetate and lead citrate. A total of 1000 nucleated cells were counted per treatment. Apoptotic cells were determined using recognised criteria²² of condensed chromatin around the periphery of the nucleus, a smooth cellular outline and condensed cytoplasm. Only cells exhibiting all three characteristics were counted as apoptotic.

Results

Haematological studies

Experiment 1: Seven days study The mean body weight of control mice increased from 24.3 g (day one) to 25.4 g (day eight). The body weights of mice

treated with concentrations of 1.0, 2.0 and 3.0 mg/ml CAPS in the drinking water were similar to those of the control mice on day eight (25.1, 25.6 and 25.0 g, respectively). However, in mice given 4.0 mg/ml CAPS, mean body weight was significantly reduced compared to the controls between day three (23.7 g, $P < 0.05$) and day five (23.2 g, $P < 0.05$), but increased compared to the controls thereafter to 24.2 g on day eight ($P < 0.01$). In animals treated with 5.0 and 6.0 mg/ml of CAPS in the drinking water, mean body weights on day eight were 21.3 g and 22.3 g, respectively; these weights were significantly lower than the controls ($P < 0.001$ for both groups).

Control mice consumed an average of 4.0 ml water/day, which compared with animals given 1.0 and 2.0 mg CAPS/ml (4.0 and 3.9 ml/day, respectively). With CAPS concentrations of 3.0 and 4.0 mg/ml and above in the drinking water, there was clear evidence of lack of palatability. Mean consumptions were reduced in mice given 3.0 and 4.0 mg CAPS/ml (3.2 and 2.9 ml/day, respectively, $P < 0.001$), and the reduction was even greater in animals given 5.0 and 6.0 mg CAPS/ml (1.6 ml/day in both groups, $P < 0.001$). Calculations showed that over the seven day period of the experiment, the mean daily consumption of CAPS was 161 mg/kg body weight (1.0 mg/ml dose), and 312, 383, 479, 356 and 434 mg/kg/day at doses from 2.0–6.0 mg/ml, respectively.

Erythrocyte counts (RBC) of mice given CAPS in the drinking water at concentrations from 1.0–4.0 mg/ml were similar to the values in control mice ($10.04 \pm 0.21 \times 10^{12}/1$). However, RBC values were increased ($P < 0.001$) in mice given 5.0 and 6.0 mg/ml CAPS (10.68 ± 0.28 and $10.76 \pm 0.29 \times 10^{12}/1$, respectively). This trend, of an increasing value in the blood parameter with a higher CAPS dose, was also seen in Hb and HCT values (control Hb value, 15.7; 16.8 and 16.9 g/dl at 5 and 6 mg/ml CAPS, respectively; control HCT value 0.518; 0.551 and 0.560 l/l at 5 and 6 mg/ml CAPS respectively; all increases, $P < 0.001$). Absolute reticulocyte counts showed a general dose-related reduction with increasing CAPS concentrations. This achieved statistical significance ($P < 0.05$) in animals given CAPS at concentrations of 3.0 mg/ml and above (controls 203 ± 50 ; 3.0 mg/ml, 149 ± 36 ; 4.0 mg/ml, 107 ± 51 ; 5.0 mg/ml, 99 ± 44 ; 6.0 mg/ml, $116 \pm 48 \times 10^9/1$); however, this trend of reduced numbers was only apparent in the more mature reticulocytes. Platelet counts, and total nucleated cell counts for the femoral marrow and spleen, were unaffected by the administration of the drug. No other changes in haematological values were considered to be related to a toxic effect of the antibiotic.

Experiment 2: 30 days study The mean body weight of control mice increased from 25.1 g (day

one) to 27.7 g (day 29). In mice given CAPS at a concentration of 2.5 mg/ml in the drinking water, the body weights compared with those of the control animals (25.1 g, day one; 27.9 g, day 29). At a concentration of 4.0 mg/ml, there was a reduction in mean body weight from 25.1 g (day one) to 24.4 g (day two) but thereafter mean body weight increased, and from day 11 (26.1 g) to day 29 (27.0 g) the weights were equal to those of the control animals.

The mean water consumption of control mice over the period of the experiment was 4.2 ml/day. For mice consuming drinking water containing 2.5 mg/ml CAPS, there was an indication of a lack of palatability, the mean consumption being reduced to 3.8 ml/day ($P < 0.05$). The mean consumption over the experimental period for animals administered 4.0 mg/ml CAPS was 3.0 ml/day, a significant decrease ($P < 0.001$). Calculations of daily CAPS ingestion over the period of the experiment were 380.9 and 438.0 mg/kg body weight per day, for mice consuming the drug at concentrations 2.5 and 4.0 mg/ml, respectively.

The numbers of RBCs in mice consuming 2.5 mg/ml CAPS compared with those in the control animals. However in those animals consuming 4.0 mg/ml, the RBC number was increased on day five (controls, 9.76 ± 0.20 ; 4.0 mg/ml, $10.33 \pm 0.38 \times 10^{12}/l$, $P < 0.05$): after day five, the RBC numbers compared with those in the control animals. The numbers of WBCs, neutrophils and platelets in mice consuming 2.5 and 4.0 mg/ml CAPS were the same as the controls. The absolute number of reticulocytes in animals consuming 2.5 mg/ml CAPS was decreased compared to that in control animals at day five (controls 244 ± 37 ; 2.5 mg/ml, $171 \pm 40 \times 10^9/l$, $P < 0.05$), but thereafter the numbers were similar to those in controls. The numbers of reticulocytes were reduced in mice consuming 4.0 mg CAPS/ml on day five (controls, 244 ± 37 ; 4.0 mg/ml, $92 \pm 18 \times 10^9/l$, $P < 0.001$), and on day 10 (control, 240 ± 12 ; 4.0 mg/ml, $169 \pm 64 \times 10^9/l$, $P < 0.05$), but then the number of reticulocytes demonstrated an 'overshoot' to $347 \pm 66 \times 10^9/l$ ($P < 0.01$) on day 15, and thereafter the numbers compared closely with the controls. Analysis of reticulocyte fluorescence ratios showed significant effects in the HFR fraction (ie the most immature reticulocytes) when expressed as absolute numbers. On days five, 20 and 30 the numbers were significantly reduced ($P < 0.05$) at each time point in animals consuming 2.5 mg/ml; in animals consuming 4.0 mg/ml, the numbers of HFRs were reduced on days five ($P < 0.001$) and 30 ($P < 0.01$), but significantly increased on day 15 ($P < 0.05$). Numbers of femoral and splenic nucleated cells in the treated groups compared with those of control animals. All other haematological parameters showed no changes considered to be related to an adverse effect of the drug.

Experiment 3: 13 months study Mean body weights of control mice increased from 29.1 g at the beginning of the experiment to 33.7 g (day 64), 36.1 g (day 113), 40.3 g (day 215), 45.5 g (day 313) and 51.1 g (day 377). The body weights of mice administered 4.0 mg/ml CAPS in the drinking water were the same as the control animals over the 13 month period of the experiment. At the beginning of the study (day four), control mice consumed an average of 4.0 ml of water per day, decreasing to 3.8 ml (day 64), 3.7 ml (day 112), 3.4 ml (day 217), 3.9 ml (day 315), and 3.7 ml (day 399). CAPS-dosed mice consumed less water than the control animals; day four, 2.7 ml; day 64, 2.9 ml; day 112, 2.9 ml; day 217, 2.8 ml; day 315, 2.5 ml and day 399, 2.4 ml. Calculations of CAPS ingestion showed that in the initial period (up to day four), mice ingested 370 mg CAPS/kg body weight/day, falling to 335 mg/kg/day (day 14), 318 mg/kg/day (day 64), 316 mg/kg/day (day 112), 249 mg/kg/day (day 217), 200 mg/kg/day (day 315), and 176 mg/kg/day (day 399).

Examination of blood parameters in control and treated mice on days 55, 92, 125, 167, 299 and 400 showed no effects on RBC numbers, Hb concentration, HCT, reticulocyte numbers or other blood parameters, that could be attributed to the administration of the antibiotic.

Assay of CAP and CAPS in serum and marrow

Experiment 1: 13 month study Of 20 mice which received CAP as CAPS in the drinking water daily for 400 days, only three had detectable amounts of CAP in the serum (0.34, 0.55, 1.72 mg/L). No CAP or CAPS was detected in the serum of four mice given drinking water without CAPS.

Experiment 2: Circadian variation in CAP and CAPS concentrations in serum and bone marrow Over the 15–16 day period of the study, control mice consumed an average of 3.8 ml of water per day, whereas those given 4.0 mg/ml CAPS in the drinking water consumed 2.8 ml per day, and those given 6.0 mg/ml CAPS consumed 2.0 ml per day. The consumptions in the last two groups were significantly reduced ($P < 0.001$). Calculations show that mice consuming water containing 4.0 mg/ml of CAPS took in 466 mg CAPS/kg body weight/day, and those consuming water containing 6.0 mg/ml of CAPS took in 525 mg CAPS/kg body weight/day. During the period of blood sampling (19.00 h to 11.00 h), measurements of water consumption (pooled across the three treatment groups) showed that from 11.00 h to 19.00 h mice consumed an average of 0.3 ml of water, from 19.00 h to 00.00 h, 1.5 ml, from 00.00 h to 06.00 h, 1.0 ml, and from 06.00 h to 11.00 h, 0.4 ml.

Serum concentrations of CAP and CAPS in individual mice at 19.00 h, 00.00 h, 06.00 h and 11.00 h, are shown in Table 1 for animals given 4.0 or 6.0 mg/ml CAPS in the drinking water (3 or 4 mice at each time point). At 19.00 h, CAP/CAPS was detected in the serum of one out of eight mice, in six of six mice at 00.00 h, in two of seven mice at 06.00 h, and in two of eight mice at 11.00 h. Neither CAP nor CAPS were detectable in the bone marrow of any animal at any time. No CAP or CAPS was detectable in the serum or marrow of 16 control mice without CAPS in the drinking water.

Table 1 Serum concentrations of CAP and CAPS in individual mice treated with CAP as CAPS in the drinking water at 4.0 and 6.0 mg CAPS/ml. Groups of 3 or 4 mice were killed at 19.00 h, 00.00 h, 06.00 h or at 11.00 h. No CAP or CAPS was detectable in the serum of 16 control mice without CAPS in the drinking water

Time of day	4.0 mg/ml CAPS in the drinking water		6.0 mg/ml CAPS in the drinking water	
	CAP (mg/L)	CAPS (mg/L)	CAP (mg/L)	CAPS (mg/L)
19.00 h	0	0	2.1	4.2
	0	0	0	0
	0	0	0	0
	0	0	0	0
00.00 h	27.7	3.9	5.4	6.7
	5.9	0	4.0	4.3
	4.6	0	3.4	3.0
06.00 h	3.1	4.4	1.9	5.8
	0	0	0	0
	0	0	0	0
	0	0	0	0
11.00 h	3.4	0	2.7	0
	0	0	0	0
	0	0	0	0
	0	0	0	0

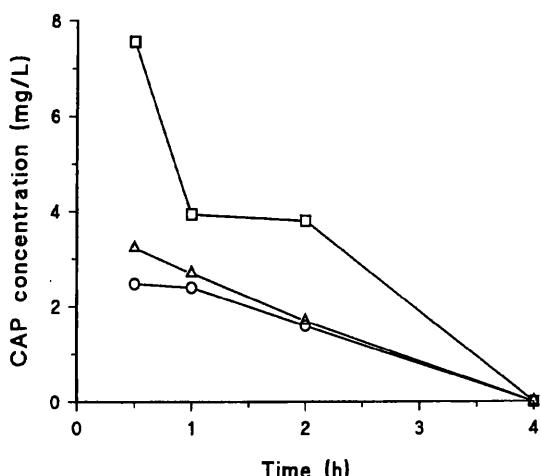


Figure 1 The kinetics of chloramphenicol (CAP) in mouse serum after the administration of chloramphenicol succinate (CAPS) by gavage at 25–100 mg/kg body weight. Symbols represent the mean values of results from five mice. \circ = 25 mg/kg; Δ = 50 mg/kg; \square = 100 mg/kg

Experiment 3: CAP administered as CAPS by gavage The clearance of CAP from the serum of animals dosed orally with CAPS at concentrations of 25, 50 or 100 mg/kg is shown in Figure 1. By four hours after dosing, CAP was cleared from the serum of all animals at all concentrations. CAPS was not detectable in the serum at any time. Likewise, no CAP or CAPS was detectable in the bone marrow at any time. No CAP or CAPS was detected in the serum or marrow of the control animals dosed with water.

Marrow cell culture

Experiment 1: Growth of marrow cells in vitro at 15–120 min following the administration CAP as CAPS A 50% reduction in the number of CFU-Es when compared with controls, after seven days in culture, was observed as early as 15 min after gavage doses of 200 mg CAPS/kg ($P<0.01$) and 100 mg CAPS/kg ($P<0.001$) (Figure 2). A significant reduction was seen at 30 min after the administration of 50 mg CAPS/kg ($P<0.01$) and at 60 min after 25 mg CAPS/kg ($P<0.05$). A small decrease in numbers of BFU-Es with increasing concentration of CAPS was seen at 120 min after gavage administration. However, this was very variable and overall there was no significant trend in dose or time response.

Experiment 2: Growth of marrow cells in vitro at 1 h to 28 days following the administration of CAP as CAPS A maximal reduction in the numbers of CFU-Es growing in culture, to 60% of the control value ($P<0.001$), was observed at 24 h after a single

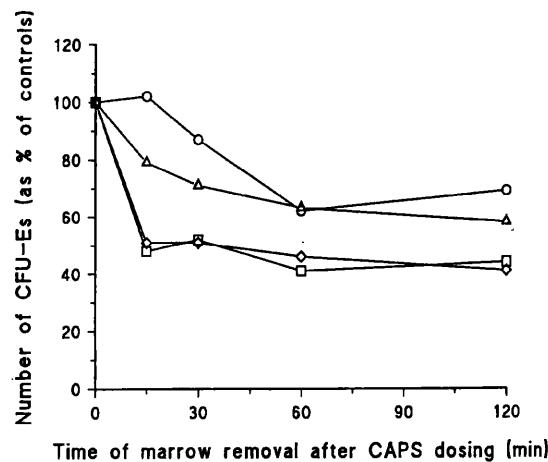


Figure 2 Growth in culture of the erythroid lineage of mouse bone marrow cells after short term exposure to chloramphenicol (CAP) *in vivo*. Administration of chloramphenicol succinate (CAPS) was by gavage at doses of 25–200 mg/kg body weight. Marrow was removed at 15, 30, 60 and 120 min after dosing. Symbols represent the mean values of results from four cultures. Data are expressed as percentages of control values. \circ = 25 mg/kg; Δ = 50 mg/kg; \square = 100 mg/kg; \diamond = 200 mg/kg

gavage dose of 50 or 200 mg CAPS/kg (Figure 3). However, by 48 h after dosing, recovery was beginning to occur, and by four days, CFU-E numbers were not significantly different from control animals. An apparent 'overshoot' in proliferative capacity of around 25% was observed between four and 28 days after administration in the 200 mg/kg group, but not in the 50 mg/kg group.

Transmission electron microscopy of marrow cells
Morphological evidence of apoptosis was seen in both erythroid and myeloid precursors in the marrow and the numbers of affected cells increased with time after CAP administration. Two hours after treatment with 200 mg CAPS/kg there were 38% more apoptotic cells in the bone marrow of treated mice than in the control bone marrow. At 24 h there were 94% more apoptotic cells in the marrow of

treated animals than in control marrow. Examples of apoptotic and normal cells are shown in Figure 4.

Discussion

The main types of haematotoxicity induced by CAP in humans, which have long been recognised, are reversible bone marrow suppression and the apparently idiosyncratic occurrence of aplastic anaemia.^{8,11} The challenge has been to develop animal models which exhibit both these effects. In the peripheral blood of treated patients, early indicators of CAP toxicity are leucopenia and thrombocytopenia. This effect was not observed in our mice treated with CAP administered as CAPS, by gavage or in the drinking water, despite the gavage dose being in the therapeutic range normally used in the treatment of infections in humans and the total daily intake of those mice receiving CAPS in the drinking water being three to five times greater than a high therapeutic dose in man.

In reversible bone marrow depression induced by CAP, the expected changes in peripheral blood parameters would be an initial reduction in Hb concentration, HCT and RBC numbers, in association with a decrease in the absolute number of reticulocytes. In Experiment 1 (seven day study), where the higher concentrations of CAPS were administered (5 and 6 mg/ml) in the drinking water, there was a dose-related increase in Hb concentration, HCT and RBC numbers, and a decrease in absolute reticulocyte numbers. There was no effect on total WBC, neutrophil or platelet numbers. In Experiment 2 (30 day study), in mice given 4.0 mg/ml CAPS in the drinking water, there were no statistically significant changes in Hb or HCT, but an early response was an increase in RBC numbers on day 5 which quickly returned to normal.

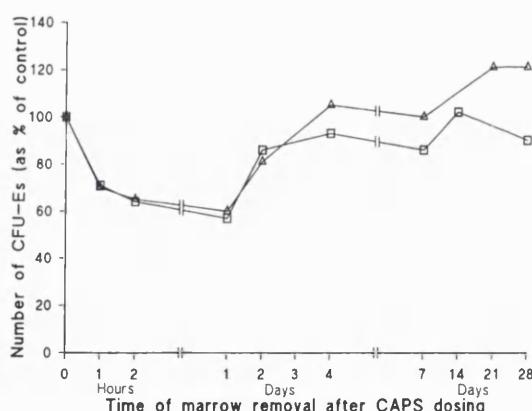


Figure 3 Growth in culture of the erythroid lineage of mouse bone marrow cells after long term exposure to chloramphenicol (CAP) *in vivo*. Chloramphenicol succinate (CAPS) was administered by gavage at doses of 50 and 200 mg/kg body weight. Marrow was removed at 1 h and 2 h, and 1, 2, 4, 7, 14, 21 and 28 days after dosing. Symbols represent the mean values of results from four cultures. Data are expressed as percentages of control values. Δ = 50 mg/kg; \square = 200 mg/kg

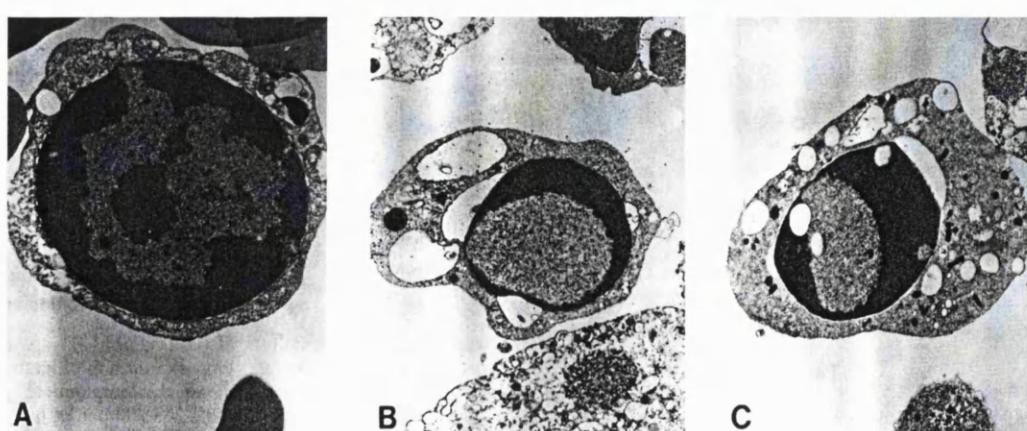


Figure 4 Apoptosis in mouse bone marrow cells at 24 h after treatment with 200 mg/kg chloramphenicol succinate (CAPS) by gavage. (A) Normal erythroid progenitor cell, $\times 12\,600$. (B) Apoptotic erythroid progenitor cell, $\times 10\,800$. (C) Apoptotic myeloid progenitor cell, $\times 10\,800$

Reticulocyte numbers were also reduced initially but returned to those close to the controls after an 'overshoot' response on day 15.

In the present experiments, which followed the regime of Morley *et al.*¹⁶ it became apparent that inclusion of CAPS in the drinking water made the water unpalatable, possibly due to the extreme bitterness of the compound. The overall effect was thus a significant dose-related reduction in water consumption and body weight. In Experiment 1, the early rise seen in Hb, HCT and RBC parameters, accompanied by a decrease in reticulocytes, contrasts with the expected pattern of a developing anaemia, and similarly with the early changes in Experiment 2. It is likely therefore that these changes are associated with mild haemoconcentration and dehydration caused by a decreased water consumption and body weight reduction, and are not the result of any effect of CAP on haematopoiesis.

A lack of significant effect of CAP on peripheral blood parameters in laboratory animals has been reported.²³⁻²⁵ A reduction in reticulocyte numbers has been seen after subcutaneous administration of high concentrations of CAP.^{26,27} Such responses in peripheral blood have been reported in laboratory rodents receiving limited amounts of food and water.^{28,29} Zaporowska and Wasilewski,³⁰ and Maejima and Nagose³¹ reported an increase in Hb, HCT and RBC parameters in starved rats with decreased water intakes. Similar increases in these parameters in rats on reduced dietary intakes over a period of 210 days were described by Schwartz *et al.*²⁸

In experiments 1, 2 and 3, CAPS ingestion ranged from 160–440 mg/kg/day but evidence of marrow depression was not seen. Firkin *et al.*²⁷ administered CAP to C57BL × CBA mice at 600 mg/kg every 8 h for 5 days by subcutaneous injection. A reduction in reticulocyte numbers occurred, but there were no significant changes in erythrocyte parameters. Similarly, Hara *et al.*²⁶ injected CAPS subcutaneously at an estimated dose of 730 mg/kg/day for 5 days to C57BL mice and reported a decrease in reticulocyte numbers, but no other haematological effects. We have recently found that doses of CAPS in the range of 1400–1700 mg/kg/day (given daily, by gavage, for 7–10 days) are required to induce a significant anaemia and reticulocytopenia in female CD-1 mice (unpublished observation).

The concentrations of CAP in the serum of the mice in the experiment lasting 400 days compare with published data,^{16,27,32} however CAP was detectable in the serum of only three of the 20 mice; 0.34, 0.55 and 1.72 mg/L respectively. A possible explanation for this seemingly inconsistent finding might be that the antibiotic breaks down in the water bottles and is therefore not available to the mice. However, assay of CAP in

the water bottles showed only a 1% breakdown over a 72 h period at a concentration of 4 mg/ml. It is more likely that CAP and CAPS concentrations in the serum had fallen toward zero by the time the mice were killed at 11.00 h on the morning of the experiments due to the drinking habits of the animals throughout the night. Further experiments established a circadian pattern where higher serum concentrations of CAP were achieved at 00.00 h, the time when the mice were most active and consuming most water; both CAP and CAPS were detectable in the serum of six mice at this time. However at 06.00 h CAP and CAPS were detectable in only two of seven mice, and in only two of eight mice at 11.00 h.

In those animals dosed by gavage with CAPS, all CAP had been cleared from the serum by four hours after administration. The kinetics of CAPS in the serum were different to those of CAP, in that no CAPS was detectable at any time, indicating a very rapid clearance. However, CAPS was detected in the 00.00 h serum sample of mice administered the prodrug in the drinking water. It is likely that this apparent paradox is due to small amounts of CAPS being ingested by the mice over a period of time up to the point at which they were killed. Nevertheless, at no time point and by neither route, were CAP or CAPS detectable in the bone marrow. It remained possible therefore that haematological changes induced by CAP were not observed in the peripheral blood because insufficient antibiotic reached the bone marrow for a toxic effect to be established. However this possibility was not substantiated by experiments to investigate the growth of the bone marrow cells. Culture of marrow cells from treated mice showed that the differentiation of the immature committed progenitors to form the more mature progenitors of the erythroid lineage was severely affected such that the numbers of CFU-Es present in the cultures were depressed as early as 15 min after a gavage dose of 50, 100 or 200 mg/kg. The marrow demonstrated evidence of recovery after 48 h, showing an overshoot in proliferation at 200 mg/kg, the highest dose. It is therefore incorrect that no toxic effect was established in the marrow, but rather than the time scale of haematopoiesis, and the ability of the stem cells to make good a toxic insult by enhanced proliferation, masked any effect which may have been distinguishable in the peripheral blood.

In contrast to the effects we have reported on haematopoietic cells exposed *in vitro* to CAP,¹² little effect was observed in the present study on the differentiation of stem cells to form very early committed progenitors. There was no significant change in numbers of BFU-Es at any time. These observations suggest that the administration of CAP to mice *in vivo* causes the death of early committed progenitors i.e. BFU-E, so that they do not differ-

entiate to form CFU-Es and thus numbers of CFU-Es decrease. However it does not affect either the differentiation of stem cells to form BFU-Es or the replication of pluripotent stem cells, thus the number of BFU-Es in the culture remain at a constant level. The survival, differentiation, proliferation and development of haematopoietic precursor cells are influenced by colony stimulating factors.³³ However the enhancement of cell survival is distinct from the stimulation of proliferation. Vaux and coworkers³⁴ have shown that the induction and expression of the bcl-2 gene suppresses the death of interleukin 3-deprived cells but does not produce proliferation of stem cells. The data reported here indicate that CAP administered to mice has effects on bone marrow cell survival during differentiation, but not on the proliferation of stem cells. Therefore our model at this stage is one of bone marrow suppression rather than AA. The *in vitro* studies reported by ourselves¹² show effects on both differentiation and proliferation of stem cells. In those studies, cells were exposed *in vitro* to therapeutic amounts of CAP for variable lengths of time. We suggest therefore that it is possible to develop an *in vivo* model for AA in the mouse if conditions can be established where CAP reaches and remains in the bone marrow for a sufficiently prolonged period of time.

The morphology of the cells affected by the administration of CAP was distinctive, and similar to that reported for apoptotic death in haematopoietic cells.³⁵ This appears to be the first report of apoptotic changes in marrow cells induced *in vivo* by CAP. In the older literature on CAP the vacuolization of erythroid precursors from treated

patients was widely reported^{5,36,37} and this effect was seen here in the treated mice (Figure 1). The term 'apoptosis' was not coined until 1972.³⁸ Initiation of apoptosis has been implicated in the pathogenesis of AA³⁹ and in the 'ineffective haematopoiesis' of myelodysplastic syndromes.⁴⁰

The studies reported here suggest that the treatment of normal mice with human therapeutic doses of CAP administered by gavage as its succinate ester reduces the ability of the bone marrow to differentiate, but does not effect proliferation, and that the marrow cells are dying due to induced apoptosis. These lesions are occurring at concentrations of CAP and CAPS in the bone marrow which are undetectable by hplc. This mouse model mimics the bone marrow depression seen in patients treated with CAP and may be developed further, on the basis of data from *in vitro* experiments, to provide a model for AA. Experimentation on this issue is now in progress.

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Research Section

An assessment of chloramphenicol and thiamphenicol in the induction of aplastic anaemia in the BALB/c mouse

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Abstract—The potential of the antibiotics chloramphenicol succinate (CAPS) and thiamphenicol (TAP) to induce aplastic anaemia in the female BALB/c mouse was investigated. CAPS was administered at 2000 mg/kg, and TAP at 850 mg/kg, daily by gavage, for 17 days. At 1, 13, 22, 41, 98 and 179 days after the final dose of each antibiotic, mice ($n = 4$ or 5) were sampled for haematological examination and haematopoietic stem cell assays. Both CAPS and TAP induced significant reductions in red blood cell count, haematocrit and haemoglobin values at day 1 post dosing; counts of colony-forming units-erythroid and colony-forming units-granulocyte-macrophage, were similarly significantly decreased at this time. All these reduced parameters returned towards normal at days 13 and 22. At days 41, 98 and 179, results for all haematological values and stem cell assays in both CAPS- and TAP-treated mice compared with the controls; there was no evidence of a reduction in peripheral blood values or bone marrow parameters at the later sampling points, as would be expected in a developing or overt bone marrow aplasia. We therefore consider that the administration of CAPS and TAP, which have been associated with the development of aplastic anaemia in man, induce a reversible anaemia, but not a chronic bone marrow aplasia, when given at haemotoxic dose levels for 17 days in the BALB/c mouse. © 2000 Elsevier Science Ltd. All rights reserved

Keywords: haemotoxicity; myelotoxicity; chloramphenicol; thiamphenicol; aplastic anaemia; BALB/c mouse.

Abbreviations: BU = busulphan; CAP = chloramphenicol; CAPS = chloramphenicol succinate; CFU-E = colony-forming units-erythroid; CFU-GM = colony-forming units-granulocyte-macrophage; EPO = erythropoietin; FNCC = femoral marrow nucleated cell count; Hb = haemoglobin; HCT = haematocrit; HFR = high fluorescence reticulocytes; IMDM = Iscove's modified Dulbecco's medium; LFR = low fluorescence reticulocytes; LUC = large unstained cells; lymph = lymphocytes; MCH = mean cell haemoglobin; MCHC = mean cell haemoglobin concentration; MCV = mean cell volume; M:E = myeloid:erythroid; MFR = mid fluorescence reticulocytes; MNC = mononuclear cells; mono = monocytes; Neut = neutrophils; PBS = phosphate buffered saline; plt = platelets; RBC = red blood cells; TAP = thiamphenicol; WBC = white blood cells.

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INTRODUCTION

Chloramphenicol (CAP) is a highly active, broad-spectrum antibiotic produced by *Streptomyces venezuelae*. It is bactericidal, has excellent pharmacokinetic properties (Holt *et al.*, 1993, 1995), and rapidly penetrates into cerebrospinal fluid; CAP can be administered by the oral, intravenous and intramuscular routes, and is relatively cheap. CAP (=chloromycetin) was first isolated in 1947, and was used clinically the following year in the treatment of typhus fever (Volini *et al.*, 1950). The antibiotic became widely used in the treatment of serious infections, including typhoid fever and other forms of salmonellosis, in severe infections due to *Haemophilus influenzae*, particularly meningitis, and in other life-threatening infections of the central nervous system and respiratory tract (Holt *et al.*, 1993; Parfitt, 1999). CAP is still used extensively in many parts of the developing world (Durosinni and Ajaya, 1993; Kumar and Verma, 1993; Kushwaha *et al.*, 1994; Trevett *et al.*, 1992). The therapeutic dose is generally 50 mg/kg daily, in divided doses, although higher levels have frequently been used; the time periods of treatment vary, but are often 10–15 days (Chaplin, 1986). CAP is still widely used in the UK and USA, in the topical treatment of eye and ear infections (Dollery, 1999; Fraunfelder *et al.*, 1993; Parfitt, 1999).

However, CAP is haemotoxic in man, inducing three major effects on the blood (FAO/WHO, 1988; IARC, 1990; Young and Alter, 1994). First, a frequently occurring, mild anaemia with reticulocytopenia, sometimes seen in conjunction with leucopenia and thrombocytopenia (Yunis and Bloomberg, 1964). This effect is dose related, develops during drug treatment, and is rapidly reversible following withdrawal of the drug (Best, 1967; Krakoff *et al.*, 1955; Scott *et al.*, 1965). The marrow generally shows normal cellularity, and an increased myeloid: erythroid (M:E) ratio, with decreased numbers of late erythroid cells (Chaplin, 1986; Rubin *et al.*, 1958; Yunis and Bloomberg, 1964). The second haemotoxic effect of CAP in man is aplastic anaemia (AA) (Hugley *et al.*, 1961; Rich *et al.*, 1950; Wallestein *et al.*, 1969; Welch *et al.*, 1954). This toxicity is relatively rare, but is evident in the blood as severe pancytopenia. CAP-induced AA is not related to the dose of the drug, develops weeks or months after treatment, and is often irreversible and fatal; the marrow is acellular or hypocellular (Yunis, 1978; Yunis and Bloomberg, 1964). The third haemotoxicity in man is leukaemia (Brauer and Dameshek, 1967; Chaplin, 1986; Cohen and Creger, 1967; FAO/WHO, 1988). It is considered that there is evidence for the carcinogenicity of CAP in humans, and that AA is related to the occurrence of leukaemia (IARC, 1990; Parfitt, 1999).

We have recently developed a mouse model of the reversible, dose-dependent anaemia seen in man, with chloramphenicol succinate (CAPS) administered to the female CD-1 weanling mouse, at 1400

mg/kg daily, by gavage, for 10 days (Turton *et al.*, 1999; Yallop *et al.*, 1998). Also, for some time we have been attempting to develop a mouse model of CAPS-induced AA in the busulphan (BU)-pretreated B6C3F₁ mouse (Andrews *et al.*, 1993, 1998; Holt *et al.*, 1998), based on dosing regimens reported by Morley (Morley and Blake, 1974ab; Morley *et al.*, 1975, 1976). The protocols of Morley involve the administration of BU on four, 2-weekly occasions to female Swiss or BALB/c mice, followed by the continuous administration of CAPS in the drinking water. However, using this regimen, we were not able to demonstrate that CAPS induces AA, or indeed any haemotoxic effects. It would appear that several workers have also examined the haemotoxicity of CAP in the BU/CAP mouse model of Morley *et al.* (1976), but their results, similarly, do not appear to closely parallel Morley's findings. Indeed, several subsequent reports have considered that no suitable or reliable laboratory animal model of AA existed (Appelbaum and Fefer, 1981; FAO/WHO, 1988; Haak, 1980; Vincent, 1986; Young and Maciejewski, 1997).

Although there is a view that rodents are not susceptible to CAP-induced myelotoxicity (Chaplin, 1986), having developed a mouse model of the human dose-related reversible anaemia (Turton *et al.*, 1999; Yallop *et al.*, 1998), we wished to investigate whether CAPS, when administered at haemotoxic dose levels (2000 mg/kg for 17 days), induced AA in the BALB/c mouse, with the animals observed for a protracted period after drug administration (6 months). Thiamphenicol (TAP), introduced as an alternative to CAP, was initially promoted as not inducing AA (Keiser, 1974; Young and Alter, 1994; Yunis, 1978); however, cases of AA subsequently emerged in patients treated with TAP (Cornet *et al.*, 1974; De Renzo *et al.*, 1981; Roussey *et al.*, 1978). We had conducted preliminary investigations on the toxicity of TAP in the mouse and rat (Havard *et al.*, 1999b) and included the antibiotic in the present study for comparative purposes, administered at an approximately equal haemotoxic dose level (850 mg/kg) to CAPS; a prefatory report has been published (Robinson *et al.*, 1999). However, the haematological response to CAPS and TAP, when given for 17 days at haemotoxic levels, was less marked than expected; a "tolerance" to the toxicity of both drugs appeared to be developing. Accordingly, a "follow-up" experiment was carried out to investigate this response; a preliminary report has been published (Havard *et al.*, 1999a).

MATERIALS AND METHODS

Animals

Female, weanling BALB/c mice (A. Tuck and Son Ltd, Essex, UK), were caged in groups of five to 15 on wood shavings with diet (Rat and Mouse No.1, SDS Ltd, Essex), and mains drinking water, *ad lib.* A

temperature of 19–22°C was maintained, with a relative humidity of 45–65%, and a light/dark cycle of 12 hr (lights on at 07.00 hr). Animals were acclimatised for at least 7 days before the start of each experiment; they were observed daily for signs of ill-health during the time of drug administration, and two or three times each week in the post-dosing periods. Body weights were determined daily, or two or three times each week, or at appropriate times. All animal procedures followed the Home Office (1989) "Code of Practice for the Housing and Care of Animals used in Scientific Procedures".

Administration of drugs

Solutions of chloramphenicol succinate (CAPS; Sigma Chemical Co Ltd, Poole, Dorset, UK) in distilled water, or suspensions of thiamphenicol (TAP; Sigma) in vegetable oil, were administered to mice by gavage at a constant dose volume of 10 ml/kg body weight. The suspension of TAP was sonicated for 5 min at 25°C after preparation. The drug was kept in suspension during the dosing period by constant mixing on a rotary mixer; a wide-bore gavage needle was used to administer the suspension. Control animals were given distilled water or vegetable oil at the above dose volume.

Haematological measurements

Mice were killed by ip injection of pentobarbitone sodium (Sagatal, Rhône Mérieux Ltd, Harlow, Essex, UK) and blood removed from the right ventricle following a thoracotomy incision. Blood (0.5 ml) was anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacriston, Durham, UK). The contents of the right femur were aspirated into 1.5 ml of phosphate buffered saline (PBS) to prepare a marrow cell suspension; a marrow smear was prepared from the contents of the right tibia. The left femur was removed and placed in sterile PBS for the preparation of marrow samples for *in vitro* culture. The sternum was removed and placed in 10.5% phosphate buffered formalin fixative, and sections prepared and stained with haematoxylin and eosin for histological examination.

Blood samples and bone marrow suspensions were analysed with a Technicon H*1 haematology analyser with mouse-specific software (Bayer Diagnostics UK Ltd, Newbury, Bucks, UK), as described previously (Andrews *et al.*, 1993, 1998). Reticulocyte analysis was performed with a Sysmex R-1000 (Toa Medical Electronics, Milton Keynes, Bucks, UK), with voltage gain adjusted optimally for mouse samples; three equal divisions of the total number of reticulocytes gave the percentage low (L), mid (M) and high fluorescence ratio reticulocytes (HFR). For the femoral marrow cell suspensions in PBS, the total nucleated cell count (femoral nucleated cell count; FNCC) was obtained from the basophil channel of the H*1. Tibial marrow smears were stained with May-Grünwald-Giemsa and differential counts performed by eye on 200 cells to give the myeloid:erythroid (M:E) ratio. Sternal marrow sections were assessed

for cellularity, myelopoiesis, erythropoiesis, megakaryopoiesis and the presence of fat.

Haemopoietic stem cell assays

Bone marrow sample preparation

Using sterile techniques, the left femur was cleaned of extraneous tissue, and the proximal and distal epiphyses removed. The marrow was aspirated into 1.0 ml Iscove's modified Dulbecco's medium (IMDM) containing 10% foetal calf serum (FCS) and placed on ice. To remove erythrocytes, the sample was diluted with 1.0 ml IMDM, added to 6 ml Lymphoprep (Nycomed Pharma AS, Oslo, Norway), centrifuged at 400 g for 30 min at room temperature, and the mononuclear cells (MNC) removed from the interface and resuspended in fresh IMDM.

Committed bone marrow progenitor assays

The technique followed that of Miller *et al.* (1996). Briefly, the cell count of the suspension was adjusted to 10⁴ cells/ml with IMDM, and 300 µl bone marrow derived MNC added to 3 ml MethoCult GF M3434 medium (Metachem Diagnostics Ltd, Northampton, UK); the tubes were vortexed, allowed to stand for 5 min at 37°C, dispensed into six-well plates, and cultured in a 5% CO₂ incubator at 37°C. Each bone marrow sample was assayed in duplicate or triplicate. Colony-forming units-erythroid (CFU-E) and colony-forming units-granulocyte-macrophage (CFU-GM) were counted after 12 days of culture.

Statistical analysis

Treated and control groups were compared using Student's *t*-test for unpaired samples.

Experimental design

Experiment 1: 28-wk study

BALB/c mice (n=4 or 5) were gavage dosed daily with water, or CAPS at 2000 mg/kg, or vegetable oil, or TAP at 850 mg/kg, for 17 days. At day 1, and on days 13, 22, 41, 98 and 179 after the final dose of each antibiotic, samples were taken for haematology and haemopoietic stem cell assays.

Experiment 2: 21-day study

BALB/c mice (n=4, 5 or 6) were gavage dosed daily with water, or 1250 or 2500 mg/kg CAPS for 5, 11, 15 or 20 days. At one day after the final dose (i.e. on days 6, 12, 16 and 21, respectively) mice were killed from each dose group and samples taken for haematological investigation; a group of untreated (control) mice were also sampled on day 1, the first day of CAPS administration.

RESULTS

Experiment 1: 28-wk study

In the investigation evaluating CAPS, control female BALB/c mice increased in mean body weight

from 19.0 g at the beginning of the 17-day dosing period, to 20.3 g at the end; in mice dosed with CAPS at 2000 mg/kg there was a slight decrease in body weight during the dosing period, from 19.1 g to 17.8 g ($P < 0.05$). However, the reduction in body weight in CAPS-dosed animals was rapidly reversed after drug treatment, and on day 11 after the final CAPS dose, control mice weighed 21.2 g and CAPS-treated mice 21.0 g. Thereafter, the body weights of both groups were similar and at the final autopsy (day 179) control mice weighed 24.2 g and CAPS mice 23.6 g. Mice given CAPS showed no clinical signs of toxicity during the period of antibiotic administration, and at post-mortem examination at one day after the final CAPS dose, and at all subsequent autopsies, there were no gross internal abnormalities.

In the investigation with TAP, control mice were 18.9 g in mean body weight at the beginning of the dosing period, and increased to 21.4 g at the end; in mice dosed with TAP at 850 mg/kg, over the same period, there was a reduction in mean body weight, from 19.2 g to 16.8 g ($P < 0.001$). However, this loss was rapidly made up in the post-dosing period and at day 22 after the final TAP dose, control mice weighed 21.8 g and TAP mice, 22.2 g. After this time, the mean body weights of the two groups were similar, increasing to 24.0 g (control) and 25.2 g (TAP) at the final autopsy (day 179).

Mice dosed with TAP showed some signs of toxicity during the period of dosing: there was a slight loss of condition with, on occasion, a deterioration in the quality of the fur, wetness or staining of the urogenital region and a slightly swollen abdomen. At the post-mortem examination at one day after the final dose of TAP, the small and large intestines of the treated mice were slightly red and swollen, and the intestinal contents were more fluid and contained some gas. The faecal pellets were of normal consistency and colour. At the autopsy at day 13 post dosing, the intestines of the TAP-dosed mice were slightly red and enlarged, but there were no other changes; at all subsequent autopsies there were no macroscopic internal abnormalities.

Haematological data is presented in Table 1 for CAPS-treated mice. CAPS induced a significant reduction in red blood cell (RBC) count, haematocrit (HCT), haemoglobin (Hb), mean cell volume (MCV) and mean cell haemoglobin (MCH) values at day 1 after the final dose of antibiotic. At day 13, these parameters in CAPS-dosed animals were returning towards control values, and by day 22 the values were similar to those of the control mice, except in the case of Hb. Total reticulocyte counts, and values for L, M and HFR, were normal immediately after the CAPS dosing period. The white blood cell (WBC) count and lymphocyte count were slightly but not significantly raised at day 1 post dosing in CAPS-treated mice, but the neutrophil count was significantly increased at this time. The platelet count

was significantly raised at day 1 in CAPS-treated mice and this increase above the control value was still evident at day 22. Counts of CFU-E and CFU-GM were significantly decreased immediately post-dosing (day 1) and at day 13; the counts then showed a return towards normal at day 22.

At day 41, 98 and 179 after the dosing period, the values of all peripheral blood parameters in CAPS-treated mice, in general, were similar to the controls; there was no evidence of significant reductions in any parameter in the later stages of the experiment; the femoral nucleated cell counts (FNCC), and counts of CFU-E and CFU-GM, were normal.

The results for TAP-treated mice are given in Table 2. At day 1 after the final dose of the antibiotic, RBC, HCT and Hb were significantly decreased, but at day 13 the values were returning towards normal, and at day 22 the results for TAP-treated mice compared with the control animals. Total reticulocyte counts in TAP-treated mice were slightly lower at day 1 (NS), and the MFR and HFR counts were significantly reduced at this time; values for these reticulocyte parameters were normal at day 13. The WBC and neutrophil counts were normal in TAP-dosed animals at day 1, but lymphocyte and monocyte counts were decreased. The mean platelet count in control mice at day 1 post dosing was 912, with individual counts ranging from 829 to $1007 \times 10^9/\text{litre}$; the individual counts of 5 TAP-treated mice were 375, 1091, 1661, 1991 and $3222 \times 10^9/\text{litre}$. This increase in the platelet count in TAP-dosed animals was also evident at day 13 post dosing ($P < 0.05$). The FNCC was decreased at day 1 in TAP-treated mice, but was normal at day 13 and 22. The results for colony forming assays showed close parallels with the data from CAPS-treated mice; both CFU-E and CFU-GM were significantly decreased at day 1 and 13 post dosing, with a return towards control values at day 22.

In general, the results for all blood parameters in TAP-treated mice at days 41, 98 and 179 after dosing were comparable to those of the control animals. Again, as with CAPS, there was no evidence of significant decreases in any peripheral blood parameters in the later stages of the study in TAP-treated mice, and FNCC, and counts of CFU-E and CFU-GM, were all similar to the values in control animals.

Marrow smears were examined and M:E ratios calculated for control, CAPS- and TAP-treated mice; sternal marrow sections were assessed using a six-point scale (Table 3). At day 1 post dosing, M:E ratios were increased from a mean of 0.85:1 (control) to 3.47:1 and 21.79:1 in CAPS- and TAP-treated mice, respectively; the highest individual M:E ratios were 12.18:1 (CAPS), and 26.17:1 and 49.67:1 (TAP). The appearance of marrow films from control, CAPS- and TAP-treated mice at day 1 are shown in Plates 1-5. Nuclear and cytoplasmic vacuolation of early myeloid cells was evident in two of five TAP-treated mice (Plate 4), but this change was not seen in

Table 1. Haematological results^a from female BALB/c mice dosed with CAPS daily for 17 days at 2000 mg/kg and sampled at 1, 13, 22, 41, 98 and 179 days after the final dose^b

	Day										Chloramphenicol and thiamphephenicol haemotoxicity	
	1		13		22		41		98			
	Control	CAPS	Control	CAPS	Control	CAPS	Control	CAPS	Control	CAPS	Control	CAPS
RBC	10.28 (0.21)	9.28 (0.33)***	10.70 (0.33)	10.26 (0.23)	10.47 (0.27)	10.48 (0.18)	10.62 (0.21)	10.35 (0.21)	10.80 (0.12)	10.20 (0.75)	10.09 (0.82)	10.34 (0.17)
HCT	0.522 (0.008)	0.445 (0.025)***	0.545 (0.013)	0.528 (0.014)	0.533 (0.007)	0.527 (0.011)	0.548 (0.034)	0.515 (0.005)	0.538 (0.005)	0.512 (0.032)	0.502 (0.038)	0.512 (0.020)
Hb	15.5 (0.2)	13.3 (0.8)***	16.4 (0.5)	15.6 (0.3)*	15.8 (0.2)	15.4 (0.2)*	16.5 (0.5)	16.0 (0.4)	16.3 (0.3)	15.3 (1.0)	15.1 (1.2)	15.2 (0.6)
MCV	50.8 (0.3)	47.9 (1.6)**	50.9 (0.8)	51.5 (0.3)	51.0 (1.0)	50.3 (0.9)	50.6 (0.7)	49.8 (0.9)	49.9 (0.4)	50.2 (1.2)	49.8 (0.5)	49.6 (2.1)
MCH	15.1 (0.2)	14.4 (0.4)*	15.3 (0.3)	15.2 (0.1)	15.1 (0.3)	14.9 (0.2)	15.6 (0.2)	15.5 (0.05)	15.1 (0.1)	15.0 (0.2)	15.0 (0.1)	14.7 (0.6)
MCHC	29.7 (0.4)	30.0 (0.6)	30.0 (0.4)	29.6 (0.3)	29.5 (0.2)	29.6 (0.4)	30.8 (0.3)	31.0 (0.7)	30.2 (0.3)	30.0 (0.7)	30.2 (0.4)	29.7 (0.6)
Retic	261.2 (58.7)	259.4 (256.3)	300.6 (62.8)	345.8 (59.1)	273.0 (13.4)	248.5 (70.5)	234.6 (14.7)	198.0 (29.6)	224.6 (49.7)	233.3 (59.1)	281.6 (63.6)	277.1 (58.7)
LFR	154.0 (29.8)	132.3 (130.9)	168.0 (24.6)	174.2 (44.1)	151.7 (12.2)	145.1 (47.5)	128.7 (7.6)	115.4 (11.6)	131.7 (24.8)	137.4 (33.3)	162.7 (43.1)	165.8 (35.6)
MFR	84.5 (27.8)	85.0 (84.4)	95.7 (29.5)	121.7 (18.2)	92.3 (6.1)	78.1 (20.0)	72.8 (4.5)	64.2 (13.4)	72.6 (19.4)	77.8 (22.7)	96.6 (25.0)	91.9 (22.2)
HFR	22.7 (15.2)	42.1 (56.1)	37.0 (13.8)	49.9 (10.2)	29.0 (8.9)	25.3 (8.7)	33.1 (11.5)	18.4 (5.7)	20.3 (7.6)	18.2 (6.0)	22.2 (5.2)	19.5 (6.2)
Pit	933 (94)	1281 (311)*	895 (127)	1121 (75)*	844 (26)	1030 (45)***	859 (130)	955 (71)	917 (81)	795 (433)	795 (104)	578 (358)
WBC	1.2 (0.5)	2.1 (0.9)	1.6 (0.5)	1.2 (0.3)	1.7 (0.4)	1.4 (0.4)	1.8 (1.1)	1.1 (0.2)	1.4 (0.6)	1.6 (0.6)	0.9 (0.3)	0.9 (0.3)
Neut	0.27 (0.10)	0.57 (0.27)*	0.46 (0.13)	0.35 (0.10)	0.36 (0.11)	0.34 (0.14)	0.41 (0.20)	0.23 (0.02)	0.34 (0.15)	0.31 (0.12)	0.21 (0.07)	0.24 (0.13)
Lymph	0.83 (0.42)	1.42 (0.84)	1.01 (0.32)	0.75 (0.23)	1.20 (0.30)	0.97 (0.23)	1.28 (0.86)	0.80 (0.22)	0.97 (0.49)	1.13 (0.47)	0.56 (0.23)	0.59 (0.19)
Mono	0.06 (0.03)	0.06 (0.04)	0.07 (0.04)	0.04 (0.02)	0.05 (0.02)	0.04 (0.01)	0.04 (0.03)	0.03 (0.01)	0.03 (0.03)	0.05 (0.02)	0.02 (0.01)	0.02 (0.01)
LUC	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.00 (0.00)	0.01 (0.00)	0.00 (0.01)	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)
FNCC	1.24 (0.27)	1.28 (0.29)	1.00 (0.08)	1.33 (0.26)*	0.88 (0.22)	1.11 (0.07)	1.67 (0.93)	1.30 (0.09)	0.94 (0.23)	0.81 (0.13)	1.33 (0.19)	1.07 (0.35)
CFU-E ^c	61.4 (7.9)	40.5 (6.2)***	62.7 (5.5)	39.5 (3.2)***	59.5 (5.8)	48.3 (4.4)*	55.7 (3.8)	56.8 (2.5)	65.2 (4.4)	66.3 (5.0)	69.7 (3.6)	69.5 (3.2)
CFU-GM	31.8 (4.6)	19.3 (6.7)***	33.8 (4.2)	20.0 (2.7)***	30.2 (3.2)	25.3 (2.8)*	27.8 (4.0)	26.3 (3.5)	31.3 (2.6)	31.5 (3.2)	33.7 (4.9)	35.0 (2.8)

^aValues are means, SD in parentheses; n=5 for control and CAPS groups at all time points, except n=4 for control groups at day 41 and 179, and for CAPS groups at day 13 and 41; for CFU-E and CFU-GM, n=6 or 9 cultures from three mice per group; *significantly different to control animals, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^bAbbreviations and units: RBC, red blood cells, $\times 10^{12}/l$; HCT, haematocrit, l/l; Hb, haemoglobin, g/dl; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $\times 10^9/l$; LFR, MFR, HFR, low, mid and high fluorescence reticulocytes, $\times 10^9/l$; Pit, platelets, $\times 10^9/l$; WBC, white blood cells, $\times 10^9/l$; Neut, neutrophils, $\times 10^9/l$; Lymph, lymphocytes, $\times 10^9/l$; Mono, monocytes, $\times 10^9/l$; LUC, large unstained cells, $\times 10^9/l$; FNCC, femoral marrow nucleated cell count, $\times 10^7$; CFU-E, colony-forming units-erythroid, absolute number of colonies; CFU-GM, colony-forming units- granulocyte-macrophage, absolute number of colonies.

^cThe control results presented for CFU-E and CFU-GM are data from TAP control cultures (Table 2); they are included here for clarity; CFU-E and CFU-GM results from CAPS-treated mice were analysed statistically against TAP control cultures.

Table 2. Haematological results^a from female BALB/c mice dosed with TAP daily for 17 days at 850 mg/kg and sampled at 1, 13, 22, 41, 98 and 179 days after the final dose

	Day											
	1		13		22		41		98		179	
	Control	TAP	Control	TAP	Control	TAP	Control	TAP	Control	TAP	Control	TAP
RBC	10.94 (0.38)	8.76 (0.67)*	10.73 (0.57)	10.02 (0.16)*	10.54 (0.22)	10.63 (0.19)	10.35 (0.11)	10.30 (0.42)	10.41 (0.22)	10.64 (0.42)	10.16 (0.32)	10.31 (0.30)
HCT	0.537 (0.010)	0.417 (0.030)***	0.549 (0.035)	0.502 (0.043)	0.525 (0.027)	0.546 (0.017)	0.522 (0.013)	0.508 (0.012)	0.526 (0.007)	0.533 (0.024)	0.515 (0.015)	0.497 (0.013)
Hb	15.9 (0.4)	12.6 (0.9)***	16.5 (1.0)	15.2 (0.9)	15.4 (0.9)	16.1 (0.2)	15.5 (0.4)	15.4 (0.4)	15.7 (0.3)	16.2 (0.6)	15.2 (0.4)	14.8 (0.7)
MCV	49.1 (1.0)	47.7 (1.2)	51.2 (1.3)	51.0 (2.2)	49.7 (1.9)	51.3 (1.0)	50.5 (0.9)	49.4 (1.2)	50.5 (0.6)	50.1 (0.8)	50.8 (0.8)	48.2 (2.0)*
MCH	14.5 (0.2)	14.4 (0.4)	15.3 (0.4)	15.2 (0.8)	14.6 (0.7)	15.2 (0.2)	15.0 (0.3)	15.0 (0.4)	15.0 (0.2)	15.2 (0.1)	14.9 (0.4)	14.4 (0.9)
MCHC	29.6 (0.3)	30.3 (1.4)	30.0 (0.3)	29.8 (0.4)	29.2 (0.4)	29.5 (0.6)	29.8 (0.3)	30.4 (0.2)**	29.8 (0.4)	30.3 (0.4)	29.5 (0.4)	29.8 (0.7)
Retic	229.2 (81.4)	81.0 (156.4)	310.6 (47.8)	351.1 (93.6)	305.6 (23.9)	311.9 (30.6)	275.1 (66.5)	234.7 (35.9)	259.9 (51.6)	251.7 (42.1)	256.2 (34.5)	281.7 (43.1)
LFR	124.3 (26.5)	66.0 (123.3)	163.4 (17.8)	180.3 (54.8)	155.4 (12.6)	176.9 (14.6)	149.0 (28.8)	126.6 (20.9)	144.3 (28.9)	146.4 (25.6)	140.6 (16.8)	170.3 (26.6)*
MFR	79.4 (35.6)	13.6 (30.1)*	106.9 (20.4)	117.3 (33.5)	105.2 (12.2)	101.2 (17.5)	90.4 (22.6)	79.2 (12.2)	91.2 (21.2)	83.6 (15.5)	94.6 (15.3)	95.8 (20.3)
HFR	25.5 (21.5)	1.4 (3.0)*	40.2 (15.6)	53.6 (18.6)	44.9 (13.5)	33.4 (15.6)	35.7 (16.4)	28.8 (10.1)	24.4 (12.1)	21.6 (7.4)	21.0 (5.6)	15.5 (5.8)
Plt	912 (66)	1668 (1064)	870 (56)	1273 (234)*	966 (61)	838 (288)	1003 (71)	893 (61)*	938 (37)	877 (81)	883 (105)	875 (119)
WBC	1.6 (0.3)	1.0 (0.6)	1.5 (0.8)	1.2 (0.2)	1.6 (0.7)	1.9 (0.5)	1.8 (0.4)	1.3 (0.2)*	1.4 (0.1)	1.1 (0.3)	1.1 (0.2)	0.9 (0.4)
Neut	0.35 (0.06)	0.50 (0.42)	0.29 (0.13)	0.36 (0.07)	0.46 (0.27)	0.41 (0.19)	0.51 (0.17)	0.29 (0.09)	0.35 (0.06)	0.28 (0.12)	0.28 (0.07)	0.20 (0.09)
Lymph	1.14 (0.24)	0.43 (0.19)***	1.08 (0.59)	0.75 (0.10)	1.01 (0.54)	1.32 (0.28)	1.15 (0.26)	0.89 (0.14)	0.92 (0.14)	0.72 (0.19)	0.69 (0.15)	0.56 (0.33)
Mono	0.06 (0.02)	0.02 (0.02)**	0.07 (0.04)	0.04 (0.02)	0.04 (0.02)	0.05 (0.02)	0.05 (0.02)	0.03 (0.01)	0.05 (0.02)	0.05 (0.03)	0.02 (0.01)	0.02 (0.01)
LUC	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)	0.00 (0.00)	0.01 (0.01)	0.01 (0.00)	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
FNCC	1.61 (0.27)	1.01 (0.36)*	1.41 (0.28)	1.52 (0.32)	1.38 (0.13)	1.23 (0.22)	1.13 (0.23)	1.61 (0.31)*	0.86 (0.30)	0.85 (0.20)	1.41 (0.18)	1.60 (0.22)
CFU-E	61.4 (7.9)	44.7 (7.4)***	62.7 (5.5)	46.0 (4.3)***	59.5 (5.8)	51.3 (5.5)*	55.7 (3.8)	56.8 (3.3)	65.2 (4.4)	60.5 (3.7)	69.7 (3.6)	64.5 (6.1)
CFU-GM	31.8 (4.6)	21.9 (6.2)***	33.8 (4.2)	24.5 (3.2)***	30.2 (3.2)	24.0 (4.3)*	27.8 (4.0)	25.2 (3.3)	31.3 (2.6)	30.5 (4.3)	33.7 (4.9)	33.0 (2.2)

^aValues are means, SD in parentheses; n = 5 for control and TAP groups at all times, except n = 4 for control groups at day 13, 41 and 98, and n = 7 for control group at day 179; all other information as Table 1.

Table 3. Results of the examination of marrow smears (M:E ratios) and sternal marrow sections, from BALB/c mice dosed with CAPS or TAP daily for 17 days and sampled at 1, 13, 22, 41, 98 and 179 days after the final dose*

	Marrow smear; M:E ratio	Sternal marrow sections				
		Cellularity	Myelopoiesis	Erythropoiesis	Megakaryopoiesis	Fat
Day 1.	Control	0.85:1	3.00	3.00	2.00	3.00
	CAPS	3.47:1	1.75	3.00	0.50	3.25
	TAP	21.79:1	1.75	2.75	0.25	3.25
Day 13.	Control	1.37:1	2.50	3.00	2.00	3.00
	CAPS	2.38:1	3.00	3.25	2.00	3.00
	TAP	1.42:1	2.50	2.75	2.00	3.25
Day 22.	Control	1.33:1	2.50	3.00	2.00	3.00
	CAPS	1.56:1	3.00	3.00	2.00	3.00
	TAP	1.46:1	3.00	3.00	2.50	3.00
Day 41.	Control	—	3.00	3.00	2.00	3.00
	CAPS	—	3.00	2.75	2.50	3.25
	TAP	—	3.00	3.00	2.75	3.25
Day 98.	Control	—	2.50	3.00	3.00	3.00
	CAPS	—	2.00	2.75	2.75	3.00
	TAP	—	2.00	3.00	3.00	3.00
Day 179.	Control	—	2.50	3.00	3.00	3.00
	CAPS	—	2.50	3.00	3.00	3.00
	TAP	—	3.00	3.00	3.00	3.00

*Values are means: n=5 for control and treated mice for assessment of marrow smears (myeloid:erythroid ratio; M:E ratios); n=2 for controls and n=4 for treated mice for assessment of sternal marrow sections. Control marrow smears were from water (vehicle) dosed mice; control sternal sections were from oil (vehicle) dosed mice; M:E ratios were not determined from mice at day 41, 98 and 179. Sternal marrow sections were assessed for cellularity, myelopoiesis, erythropoiesis, megakaryopoiesis and the presence of fat on a 6 point scale: 0=absent; 1=very slight; 2=slight; 3=moderate; 4=marked; 5=very marked. Results were not analysed statistically.

CAPS-dosed animals nor in control mice. Fat globules were present in the marrow films of some CAPS- and TAP-treated mice at day 1 post dosing (Plates 4 and 5), but this change was not evident in the controls. At day 13 post dosing, the mean M:E ratio was slightly raised in CAPS-treated mice (2.38:1) above the control (1.37:1), but in TAP-dosed animals the ratio compared to the control result; at day 22, the M:E ratios of both drug-treated groups were similar to the controls.

The cellularity of the sternal marrow, assessed on a six-point scale, was decreased in CAPS- and TAP-treated mice at day 1 post dosing (Table 3). Myelopoiesis and megakaryopoiesis were normal at day 1 in drug-treated mice, but erythropoiesis was markedly reduced in both CAPS- and TAP-treated animals

(Plates 6, 7 and 8). However, by day 13, cellularity and erythropoiesis had returned to normal in both antibiotic-treated groups. On days 22 and 41, cellularity, erythropoiesis, myelopoiesis and megakaryopoiesis were normal in all groups. Some fat was evident in the sternal marrow of both CAPS- and TAP-treated mice at day 1, and on intermittent occasions in these groups thereafter; fat was not seen in any control mice, at any time point.

At days 98 and 179 post dosing, there was no evidence of an effect of CAPS or TAP on sternal marrow cellularity, erythropoiesis, myelopoiesis or megakaryopoiesis; both treated groups compared directly with the controls.

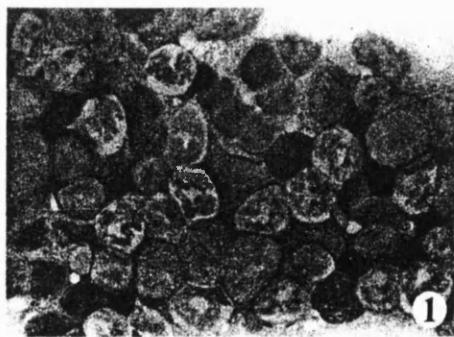


Plate 1. Romanowsky stained bone marrow smear from a control (CAPS vehicle) treated mouse, illustrating the normal distribution of hematopoietic cells. The M:E ratio is 0.78:1. Magnification $\times 3060$.

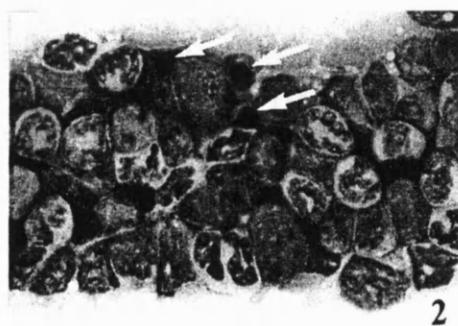


Plate 2. Romanowsky stained bone marrow smear from a mouse treated with CAPS at 2000 mg/kg for 17 days, at day 1 post treatment. The number of erythroid precursor cells is markedly decreased. A few late normoblasts (arrow) are present. The M:E ratio is 12.18:1. Magnification $\times 3060$.

Experiment 2: 21-day study

Mice were dosed with CAPS daily at 1250 and 2500 mg/kg for 5, 11, 15 and 20 days. Selected haematological results are presented in Table 4. In mice

dosed with CAPS at 1250 mg/kg for 5 days and sampled on day 6, values for HCT, Hb, MCV and MCH were significantly reduced; MCHC was increased. Total reticulocyte counts, and counts for L, M and HFR were also significantly lower in CAPS-treated mice. Values for platelets, WBC and

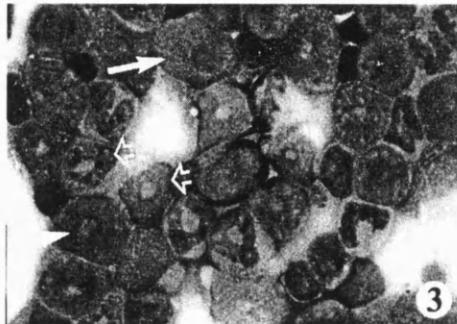


Plate 3. Romanowsky stained marrow smear from a mouse dosed with TAP at 850 mg/kg for 17 days, at day 1 post dosing; erythroid precursors are greatly reduced. Many myelocytes (arrow) and metamyelocytes (open arrow) are present. The M:E ratio is 49.67:1. Magnification $\times 3060$.

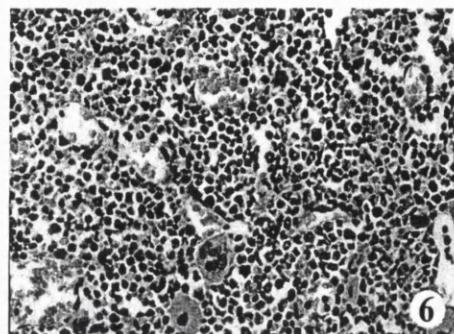


Plate 6. Sternal marrow from a control (TAP vehicle) treated mouse showing normal cellularity, and distribution of erythroid and myeloid precursors, and megakaryocytes. H & E; magnification $\times 1220$.

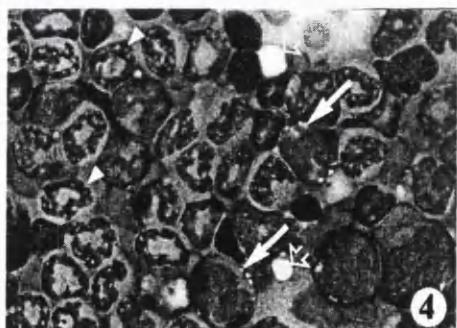


Plate 4. Romanowsky stained tibial marrow smear from a mouse given TAP at 850 mg/kg for 17 days, at day 1 post treatment. Erythroid cells are reduced. Vacuoles are present in some early myeloid and monocytic cells (arrow) and small fat globules are evident (open arrow). Many juvenile ("stab") neutrophils can also be seen (arrowhead). The M:E ratio is 4.42:1. Magnification $\times 3060$.

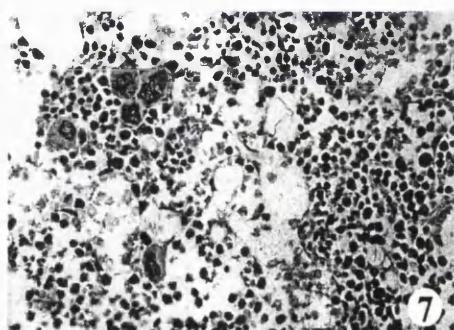


Plate 7. Sternal marrow from a CAP (2000 mg/kg/17 days) treated mouse at day 1 post dosing. Cellularity and erythropoiesis are significantly reduced, but myelopoiesis and megakaryopoiesis appear normal. H & E; magnification $\times 1220$.

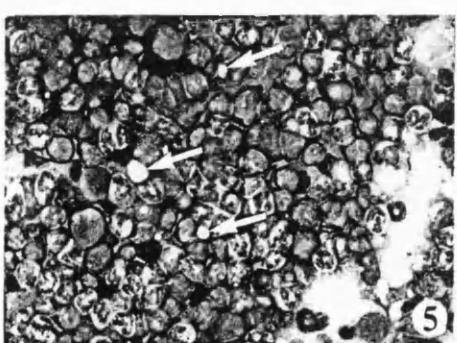


Plate 5. Romanowsky stained smear from a mouse treated with TAP at 850 mg/kg for 17 days, at day 1 post dosing, at lower power. There is a decrease in the number of erythroid precursors and fat globules are present (arrow). The M:E ratio is 26.17:1. Magnification $\times 1530$.

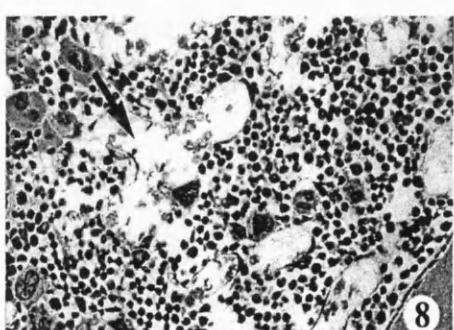


Plate 8. Sternal marrow from a TAP (850 mg/kg/17 days) treated mouse at day 1 post dosing; the marrow is hypocellular and erythropoiesis is greatly reduced. Myelopoiesis is slightly reduced but megakaryopoiesis is normal. A large sinusoid is clearly dilated (arrow). H & E; magnification $\times 1220$.

Table 4. Haematological results* from female BALB/c mice dosed with CAPS daily for 5, 11, 15 and 20 days at 1250 and 2500 mg/kg and sampled at days 6, 12, 16 and 21 respectively; control mice were also sampled at day 1, the first day of CAPS dosing

CAPS dose (mg/kg)	Day of sampling				
	1	6	12	16	21
Controls					
RBC	10.41 (0.50)	9.74 (0.30)	10.18 (0.27)	10.36 (0.70)	10.27 (0.23)
HCT	0.490 (0.027)	0.506 (0.013)	0.505 (0.026)	0.504 (0.038)	0.532 (0.016)
Hb	14.7 (0.8)	14.7 (0.2)	14.4 (0.6)	15.0 (1.4)	15.5 (0.5)
MCV	47.0 (2.1)	52.0 (0.9)	49.7 (1.9)	48.6 (1.7)	51.8 (0.7)
MCH	14.1 (0.8)	15.1 (0.3)	14.1 (0.6)	14.5 (0.08)	15.1 (0.2)
MCHC	30.1 (0.7)	29.0 (0.3)	28.4 (0.4)	29.8 (0.7)	29.1 (0.4)
Retic	353.9 (111.7)	399.4 (81.7)	403.2 (64.5)	281.1 (135.0)	276.7 (36.0)
LFM	189.0 (50.5)	187.5 (33.7)	197.1 (36.1)	171.7 (64.1)	155.0 (10.7)
MFR	120.6 (42.1)	15.2 (1.2)	16.6 (2.6)	18.5 (1.6)	83.3 (17.3)
HFR	44.3 (24.2)	6.6 (1.1)	5.7 (2.1)	2.1 (1.4)	38.4 (12.1)
Plt	594 (287)	987 (105)	871 (104)	773 (376)	842 (70)
WBC	3.3 (0.8)	1.6 (0.2)	2.5 (0.6)	1.5 (0.6)	1.7 (0.3)
FNCC	1.37 (0.25)	1.03 (0.27)	1.51 (0.11)	1.06 (0.17)	0.78 (0.37)
1250					
RBC	9.50 (0.22)	9.73 (0.69)	10.39 (0.26)	10.06 (0.13)	
HCT	0.452 (0.034)*	0.472 (0.040)	0.506 (0.016)	0.501 (0.006)**	
Hb	13.5 (0.9)*	13.8 (1.1)	14.9 (0.6)	15.2 (0.2)	
MCV	47.6 (3.1)*	48.5 (0.9)	48.7 (1.0)	49.8 (1.2)**	
MCH	14.2 (0.9)*	14.1 (0.2)	14.4 (0.3)	15.1 (0.1)	
MCHC	29.8 (0.5)*	29.1 (0.3)*	29.5 (0.2)	30.3 (0.8)*	
Retic	10.1 (3.8)***	287.2 (126.1)	469.1 (128.8)	328.7 (97.6)	
LFM	9.3 (3.5)***	179.1 (97.6)	301.0 (92.1)*	170.9 (45.5)	
MFR	6.4 (1.9)***	17.4 (0.9)	17.5 (2.1)	110.5 (34.3)	
HFR	0.1 (0.1)***	3.1 (1.2)	2.3 (1.8)	47.3 (19.8)	
Plt	1006 (499)	1395 (687)	935 (223)	971 (318)	
WBC	1.2 (0.5)	1.8 (0.4)	2.8 (0.7)*	2.2 (0.4)	
FNCC	1.07 (0.20)	1.50 (0.34)	0.92 (0.13)	0.85 (0.11)	
2500					
RBC	9.62 (0.14)	8.82 (0.41)***	8.97 (0.26)**	9.48 (0.13)***	
HCT	0.447 (0.011)***	0.420 (0.008)***	0.427 (0.009)*	0.464 (0.014)***	
Hb	13.4 (0.3)***	12.4 (0.1)***	13.0 (0.4)*	13.6 (0.2)***	
MCV	46.4 (1.2)***	47.6 (1.3)	47.6 (1.0)	48.8 (1.0)***	
MCH	13.9 (0.3)***	14.1 (0.7)	14.5 (0.2)	14.3 (0.3)**	
MCHC	30.0 (0.3)***	29.6 (0.7)*	30.4 (0.4)	29.3 (0.6)	
Retic	11.0 (2.9)***	79.7 (150.0)**	99.0 (165.8)	803.1 (544.6)*	
LFM	10.8 (3.0)***	33.5 (46.9)***	58.3 (93.2)	424.6 (216.7)*	
MFR	1.8 (1.8)***	3.5 (7.9)**	34.7 (65.9)	286.0 (261.6)	
HFR	0.0 (0.1)***	1.4 (3.2)*	5.9 (7.9)	88.5 (62.6)	
Plt	1160 (218)	1436 (756)	1460 (307)*	1209 (217)**	
WBC	1.0 (0.4)*	2.6 (3.1)	2.8 (1.8)	1.8 (0.5)	
FNCC	0.64 (0.12)	0.83 (0.29)***	0.89 (0.09)	0.93 (0.27)	

*Values are means, SD in parentheses; n = 5 for control and CAPS groups, except n = 4 for 1250 group at day 6, 12 and 21, and for 2500 group at day 21, and n = 6 for control group at day 21; all other information as Table 1.

FNCC were unaffected. In mice killed after dosing with CAPS for 11 days at 1250 mg/kg, the values for HCT, Hb, MCV, MCH and MCHC were returning towards control values; all reticulocyte counts were normal and compared with the controls. At day 11, in the CAPS-dosed animals, the mean platelet count was higher than the controls, but this increase was not statistically significant, although two animals had individual counts of 1866 and $2089 \times 10^9/\text{litre}$ (the highest count in the control group was $1005 \times 10^9/\text{litre}$). At sampling on day 16 after 15 daily doses of CAPS at 1250 mg/kg, mean values for HCT, Hb, MCV, MCH and MCHC were normal. The mean total reticulocyte count was increased in CAPS-treated mice at day 16, but this was not significant, although three mice had individual counts of 473, 521 and $642 \times 10^9/\text{litre}$; the highest individual count in the control group was $457 \times 10^9/\text{litre}$. The platelet count at day 16 in CAPS-dosed animals was similar to the control count. At day 21 in CAPS-treated

mice, some erythrocyte parameters showed decreases, in comparison with the controls, but reticulocyte and platelet counts were normal.

The administration of CAPS at 2500 mg/kg to mice for 5 days caused significant decreases in values for HCT, Hb, MCV and MCH. At day 12, after 11 doses of CAPS, the decreases in HCT and Hb, and in RBC values also, were more pronounced than at day 6. At day 16 and 21, with continued CAPS administration, HCT, Hb and RBC values were lower than the controls, but were returning towards normal. All reticulocyte parameters were significantly reduced at day 6; at day 12, although the counts were reduced, they were returning towards control values and this was also evident at day 16. On day 21, the mean total reticulocyte count in CAPS-dosed mice was significantly higher than the controls; the highest reticulocyte count in the control group was $310 \times 10^9/\text{litre}$ and the individual counts in the four mice treated with 2500 mg CAPS/kg were 405, 528, 676 and

1603×10^9 /litre. Platelet counts on day 6 and 12 were higher in CAPS-dosed mice than in the controls (NS), but on day 16 and 21 the mean counts were significantly raised. The highest individual platelet count at day 16 in the control group was 982×10^9 /litre, while for the five CAPS mice the individual counts were 1020, 1294, 1521, 1697 and 1769×10^9 /litre.

DISCUSSION

The haematological response to CAPS, administered at 2000 mg/kg for 17 days to the female BALB/c mouse, was a reduction in RBC, HCT and Hb at day 1 post dosing; these parameters then returned to normal over the following 13–41 days. Reticulocyte values at day 1 were normal. Plt counts at days 1, 13 and 22 were increased, but normal thereafter. M:E ratios were increased, and marrow cellularity and erythropoiesis were reduced at day 1 post dosing; however these parameters returned to normal at days 13/22. CFU-E and CFU-GM were decreased at day 1, returning to normal by day 41. In the later stages of the experiment, at days 98 and 179, there was no evidence in peripheral blood of a CAPS-induced decrease in the number of cells in any lineage (erythroid, WBC, platelet), no change in colony forming assays (CFU-E, CFU-GM), FNCC, and no reduction in sternal marrow cellularity or haemopoiesis. That is, there was no evidence of a CAPS-induced developing pancytopenia, or hypocellular or acellular marrow (AA). However, the reversible anaemia that was induced shows many similarities with the reversible anaemia seen in man: the murine response was a mild anaemia that developed during drug treatment and rapidly returned to normal; the M:E ratio was increased, with a decrease in erythroid sternal marrow cells. However, in contrast to the human condition, the response in the mouse was not associated with leucopenia or thrombocytopenia.

With TAP at day 1 post dosing, RBC, Hb and HCT were reduced, but returned to normal by days 13/22; reticulocyte counts were slightly lower at day 1, returning to control values at day 13. Platelet counts were higher than the controls at day 1 and 13, but normal thereafter. Results for CFU-E and CFU-GM, M:E ratios, and sternal marrow haematopoiesis, paralleled the findings with CAPS in the immediate post-dosing period. The overall pattern of changes caused by TAP, therefore (with the exception of a lymphocyte and monocyte response), compared with the CAPS-induced effects at the initial sampling times. In the later stages of the TAP study (days 98 and 179), as with CAPS, values for all observations were normal, with no indication of a developing pancytopenia, hypocellular or acellular marrow (AA). It is of interest to note that the pattern of changes in the TAP-induced reversible anaemia had similarities with the CAPS-induced effects, but was achieved with TAP administered at 850 mg/kg, compared with CAPS at

2000 mg/kg [CAPS does not show peripheral blood haemotoxicity when given at 850 mg/kg (Turton *et al.*, 1999)].

In man, drugs such as CAP only produce marrow failure relatively infrequently, and it has been proposed that some type of individual predisposition must be involved in these occasional, but severe, responses; indeed, Morley *et al.* (1976) suggested that a genetically determined abnormality of the marrow cells of particular individuals might be associated with such drug-induced conditions. To evaluate these ideas, Morley and Blake (1974a,b) and Morley *et al.* (1975, 1976) developed and assessed a model of chronic hypoplastic marrow failure (AA) in the female Swiss and BALB/c mouse, by the administration of the antineoplastic agent, BU. The essential feature of the BU-treated mouse model of Morley, is that BU produces a form of residual marrow injury which is characterised by a mild or moderate marrow failure. However, such conditions are relatively difficult to recognise in human patients, but it was suggested (Morley *et al.*, 1976) that such injury might be a factor in the predisposition of some individuals to develop AA following exposure to drugs such as CAP. To examine this proposal, Morley *et al.* (1976) evaluated the capacity of CAPS to induce chronic hypoplastic marrow failure (AA) in the female BALB/c mouse pretreated with BU. With CAPS administered in the drinking water at 5 mg/ml, the antibiotic caused a progressive fall in the number of pluripotent stem cells and granulocytic progenitor cells in BU-pretreated mice, but no effect on the marrow cells of normal (non-BU-pretreated) animals.

We have recently examined the capacity of CAPS to induce chronic hypoplastic marrow failure (AA) in the BU-pretreated female B6C3F₁ mouse (Andrews *et al.*, 1998), but in a 13-month study we were unable to show that CAPS induced AA. In our investigations, CAPS was administered in the drinking water at 4 mg/ml, and water consumption figures showed that over the course of the study the animals consumed 176–370 mg CAPS/kg/day. It is not clear why our (negative) results contrast with the positive findings of Morley *et al.* (1976). However, we have subsequently shown that at CAPS dose levels of 176–370 mg/kg/day, no haemotoxicity is caused (Holt *et al.*, 1998), and that in the CD-1 mouse, dose levels of 1400–1700 mg/kg CAPS are required to induce significant haemotoxicity (Turton *et al.*, 1999). Another factor that may be associated with our negative earlier results (Andrews *et al.*, 1998) is that, as demonstrated in Experiment 2 in the present investigations, CAPS on continued administration (e.g. in the drinking water for several months) may not induce a haemotoxic effect due to a 'tolerance' to the drug developing. Therefore, we conclude that our present findings, where CAPS administered at 2000 mg/kg for 17 days did not induce AA, support the hypothesis of Morley *et al.* (1976) that CAPS may only

induce AA if a pre-existing residual marrow injury, such as that induced by BU, is present.

Manyan and Yunis (1970) and Manyan *et al.* (1972) demonstrated that CAP inhibited mitochondrial ferrochelatase activity in dog marrow cells, with a block in haem synthesis and a depression of erythropoiesis. There was some evidence for a possible effect by CAPS on haemoglobin synthesis in the present study: MCH and MCV were significantly reduced at day 1 (Table 1), and results in Table 4 confirm this effect. There was no evidence that TAP caused a comparable change, although Yunis (1978) produced evidence that this drug also suppresses ferrochelatase activity and a block in haem synthesis.

The increase in platelet count caused by CAPS and TAP in the immediate post-dosing period, was unexpected (Tables 1, 2 and 4). However, this may relate to the frequently observed clinical finding of an inverse correlation between HCT and platelet count (Jackson *et al.*, 1974). A possible explanation may be that in anaemia, the production of erythropoietin (EPO) by the kidney increases, to stimulate erythropoiesis. The rise in platelet count may relate to the effect that EPO, as well as stimulating RBC production, also has an enhancing effect on murine megakaryopoiesis (Hunt, 1995; Sakaguchi *et al.*, 1987).

Both CAPS and TAP caused a decrease in CFU-E and CFU-GM in the immediate post-dosing period, with counts returning to normal by day 41. The decrease in the CFU-E equates with the finding of both drugs reducing erythrocyte numbers in peripheral blood. However, it is not clear why, with a decrease in CAPS-induced CFU-GM, WBC counts in blood were not similarly reduced; with TAP, both monocytes and lymphocytes were decreased, but neutrophils were unaffected. Nevertheless, Yunis (1978) discusses a comparable adverse effect of CAP in inhibiting bone marrow myeloid colony growth, and related this to the granulocytopenia sometimes observed in CAP- and TAP-treated patients. Furthermore, other workers, using other marrow depressing agents (e.g. BU), have reported reductions in marrow colony-forming assays, but relatively normal peripheral blood values (Fitchen and Cline, 1980; McManus and Weiss, 1984).

Nuclear and cytoplasmic vacuolation was evident in the early myeloid cells of the marrow smears of TAP- but not CAPS-treated mice. This finding was of interest since CAP-induced nuclear and cytoplasmic vacuolation has been reported in the marrow of patients treated with the antibiotic (Jiji *et al.*, 1963; Krakoff *et al.*, 1955; Rosenbach *et al.*, 1960; Saidi *et al.*, 1961; Scott *et al.*, 1965). This change generally involves the erythroblast (proerythroblast), but granulocytes, megakaryocytes and plasma cells were sometimes affected. The experimental induction of this effect was reported by Schrober *et al.* (1972) by the administration of CAPS twice daily at 150 mg/kg to the rat for 5 wk; vacuoles were observed in the pronormoblasts, and also in myeloblasts and

promyelocytes at the nuclear-cytoplasmic junction. The reasons for the identification of vacuoles in the myeloid cells of TAP-treated, but not CAPS-treated animals, in the present study, are unclear.

At necropsy, in TAP-dosed mice there was evidence of intestinal inflammation, with the small and large intestines being slightly red and swollen, and the contents were more fluid and contained some gas. These changes were not seen in CAPS-treated animals in the present experiment, but were observed in a previous study with CAPS in the rat (Turton *et al.*, 1999), where it was considered that such effects may be associated with a disturbance of the normal enteric flora with possible bacterial overgrowth (Morris, 1995). In a toxicity study of TAP in the rat (Ando *et al.*, 1997), caecal enlargement and gastrointestinal changes were reported, but not in a longer-term carcinogenicity study with the drug administered at a lower dose level (Kitamura *et al.*, 1997), nor in a lifetime study in the mouse with TAP administered at levels up to 250 mg/kg/day (Simpson *et al.*, 1979).

In an earlier study with CAPS in the female CD-1 mouse (Turton *et al.*, 1999), with the drug administered at 2000 mg/kg for 7 days, peripheral blood at the end of the dosing period showed a reduction in RBC count to 89.5% of the control value, a decrease in HCT to 81.4%, in Hb to 85.6%, and in the reticulocyte count to 5.3%; the platelet count was normal. However, with CAPS given at 1400 mg/kg for a period of 10 days, RBC, HCT and Hb were only slightly reduced immediately post dosing (reductions to 93.7%, 94.2% and 94.7%, respectively), and reticulocytes and platelet counts were normal (Turton *et al.*, 1999). From these experiments, it was considered that in the present investigation, a marked anaemia and reticulocytopenia immediately post dosing could be achieved by administering CAPS for 17 days at 2000 mg/kg. However, this more marked effect did not become manifest (Table 1): reductions to 90.3% of control were seen (RBC), 85.2% (HCT) and 85.8% (Hb); the reticulocyte count was normal and platelet increased. These results indicated that a "tolerance" to the haemotoxicity of CAPS was developing during the 17-day period of dosing, and that the erythrocyte and reticulocyte counts were returning to normal during the continued administration of the drug; a similar effect appeared to be occurring with TAP. A follow-up study with CAPS was therefore conducted (Experiment 2; Table 4).

The pattern of changes (Table 4) indicate that, with the continued dosing of CAPS at 1250 and 2500 mg/kg, the depressed reticulocyte counts (day 6) return to normal, followed by a return towards normal in RBC, HCT and Hb values; a "tolerance" to CAPS haemotoxicity appeared to develop. At 2500 mg/kg at day 12, RBC was 86.6% of control, with HCT, 83.2% and Hb, 86.1%.

There is evidence in the clinical literature, in patients treated with CAP, that serum concentrations of the drug decrease during a course of therapy.

Nahata and Powell (1983) demonstrated this effect in pediatric patients during a typical 10-day course of treatment. Also, Black *et al.* (1978) showed that to maintain effective blood levels of the antibiotic during a 3-wk course of therapy, the administered dose of CAP needs to be steadily increased. These findings indicate an increase in drug clearance, either directly as the unchanged molecule, or after enhanced metabolism. Further evidence suggests that interactions occur *in vivo* between CAP and other drugs, for example between pentobarbital and CAP (Adams and Dixit, 1970; Bloxam *et al.*, 1979; Krasinski *et al.*, 1982), xylazine-ketamine and CAP (Nossaman *et al.*, 1990), rifampacin and CAP (Prober, 1985), and phenytoin and CAP (Krasinski *et al.*, 1982).

A possible explanation of the present findings (Table 4) is that CAP is able to disturb its own metabolism, such that potentially toxic metabolites are not formed. For example, the induction of UDP-glucuronyl transferase would increase the formation of easily excretable glucuronide conjugates, and decrease the amount of CAP available for metabolism to proximal toxic aldehyde or nitroso derivatives (Holt *et al.*, 1993, 1995). However, this explanation must remain speculative until the metabolic pathways of CAP in the mouse are determined.

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Haemotoxicity of chloramphenicol succinate in the CD-1 mouse and Wistar Hanover rat

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- 1 Chloramphenicol has been widely used in the treatment of serious infections including typhoid fever and meningitis. However, the drug is haemotoxic in man inducing firstly, a reversible, dose-dependent anaemia which develops during treatment, secondly, an often fatal aplastic anaemia with pancytopenia and acellular marrow, and thirdly, leukaemia.
- 2 We investigated the haemotoxicity of chloramphenicol succinate (CAPS) in female CD-1 mice in repeat dose studies, to compare the response with the reversible anaemia reported in man. Studies in male Wistar Hanover rats were also carried out.
- 3 CAPS was gavaged daily to mice at dose levels from 800–2000 mg/kg for seven days. Values were significantly reduced for reticulocytes at 1700 and 2000 mg/kg, and for erythrocytes (RBC), haematocrit (HCT), and haemoglobin (Hb) at 2000 mg/kg. Platelet and white blood cell (WBC) counts were unaffected.
- 4 Mice were dosed with CAPS at 1400 mg/kg for 10 days and sampled at 1, 4 and 15 days after the last dose. At

- day 1 post dosing, RBC, HCT and Hb values were significantly reduced, but returned to normal (or above normal) by day 4 or 15.
- 5 CAPS from 2000–4000 mg/kg was gavaged to rats daily for 19 days. Hb values were significantly lower at 3600 and 4000 mg/kg; reticulocytes were not reduced. WBC and platelet counts, in general, were unaffected.
- 6 Levels of apoptosis in marrow mononuclear cells were increased in CAPS-treated mice, but not in CAPS-treated rats. Serum biochemistry parameters, in general, showed few changes of toxicological significance.
- 7 We conclude that the administration of CAPS to CD-1 mice induced haematological changes showing close parallels with the chloramphenicol-induced reversible anaemia seen in man.

Keywords: chloramphenicol succinate; haemotoxicity; mouse; rat; aplastic anaemia

Introduction

Chloramphenicol is a broad-spectrum antibiotic produced by *Streptomyces venezuelae*. It was first isolated in 1947 and used clinically the following year in the treatment of typhus fever.¹ Chloramphenicol has since been widely used in the treatment of serious infections, including typhoid fever and other forms of salmonellosis, in severe infections due to *Haemophilus influenzae*, particularly meningitis, and in other life-threatening infections of the central nervous system and respiratory tract.² The therapeutic dose is generally 50 mg/kg daily, in divided doses, although higher levels have been used. The time periods of drug treatment vary, but are often 10–15 days.³

However, chloramphenicol is haemotoxic in man, and induces three major effects.^{2–6} First, a commonly-occurring mild anaemia with reticulocytopenia, occasionally seen with leucopenia and thrombocytopenia. The condition is dose-related, develops during treatment, and is reversible. The marrow shows an increased myeloid:erythroid

(M:E) ratio with decreased numbers of late erythroid cells.^{3,4,7,8} The second effect of chloramphenicol on the blood is aplastic anaemia (AA).^{9–11} This toxicity is rare, but is evident as severe pancytopenia in peripheral blood. The effect is not dose-related, develops weeks or months after treatment, and is often fatal; the marrow is acellular or hypocellular.^{3,4,12} The third haematological toxicity in man is leukaemia.^{6,13} It is considered that there is limited evidence for the carcinogenicity of chloramphenicol in humans; also, that AA is related to the occurrence of leukaemia.⁵

We have been developing a model of chloramphenicol-induced AA in the mouse,^{14–16} based on protocols of Morley and his co-workers.^{17–19} However, recently we became interested in the dose-dependent, reversible anaemia caused by chloramphenicol in man. No animal model of this condition exists. We have now examined the haemotoxicity of chloramphenicol succinate (CAPS) in the female CD-1 mouse, in repeat dose studies, to compare the characteristics of the haematological response with those reported in man. We then went on to carry out studies, with similar objectives, in the male Wistar Hanover rat. A preliminary report has been published.²⁰

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Experiment 3 Apoptosis and serum clinical chemistry study CD-1 mice ($n=6$) were gavaged with water or 2200 mg/kg CAPS and at 18 h post dosing, blood was removed for serum preparation and clinical chemistry. Femoral marrow was aspirated into PBS for assay of apoptotic cells.

Experiment 4 CAPS acute toxicity studies BALB/c mice ($n=2$) were gavaged with a single dose of CAPS at increasing 500 mg/kg dose levels from 3000–5500 mg/kg, and from 6000–7000 mg/kg. TO mice (32 g; $n=4$) were gavaged with a single dose of CAPS at increasing 500 mg/kg dose levels from 3500–7500 mg/kg. Animals were closely observed for signs of toxicity. Mice which became ill, and judged as not likely to recover, were immediately killed.

Rat studies

Animals, administration of CAPS, sampling and analysis Outbred, male, Wistar Hanover rats (Harlan UK Ltd), approximately 130 or 340 g, were housed in groups of three to six and maintained as described for mice. CAPS was administered by gavage at a constant volume of 10 ml/kg body weight; control rats were dosed with the same volume of distilled water. Animals were killed as outlined for mice, and blood removed from the abdominal aorta. Blood samples, marrow smears and sterna were prepared as described for mice, and femoral marrow samples aspirated into 5 ml of PBS. For coagulation screening tests, nine volumes of blood were added to one volume of 0.106 M-trisodium citrate solution, and the samples placed on ice until analysis. Serum samples for clinical chemistry, and femoral marrow samples for apop-

tosis studies, were prepared as set out for mice, but marrow samples were flushed into 1.5 ml PBS. Analysis of all samples was as described for mice. Coagulation screening tests were performed as follows: for the Thrombotest assay, reagents and coagulometer from Nycomed Ltd (Birmingham, UK) were used, with procedures described previously;^{21,22} for prothrombin time (PT) analysis, rabbit brain thromboplastin/CaC1₂ (Diagen Ltd, Thame, Oxon, UK) was used and assayed on a CA5000 Automated Coagulation Analyser (Sysmex, Milton Keynes, Bucks, UK); for activated partial thromboplastin time (APTT) analysis, 'Automated' reagent (Micronised Silica Activator, Orgonon Teknika Ltd, Cambridge, UK) was used and assayed by CA5000; for fibrinogen analysis, the Klauss method was used, with fibrinogen (thrombin) reagent (Immuno Ltd, Newbury, Berks, UK), and assayed by CA5000. Analysis of serum clinical chemistry parameters was as indicated for mice but included assays for glutamate dehydrogenase (GLDH), lactic dehydrogenase (LD), creatine kinase (CK), urea, creatinine, Na, K, Ca, Cl, phosphate, glucose, triglycerides and cholesterol.

Experimental design

Experiment 1 Dose response study Wistar Hanover rats (mean body weight 134 g; $n=3$ or 5) were dosed daily, for 19 days, with distilled water or CAPS at 2000, 2400, 2800, 3200, 3600 and 4000 mg/kg. Assays for haematological and coagulation parameters were carried out on day 20.

Experiment 2 Apoptosis and serum clinical chemistry study Twelve rats (mean body weight, 340 g) were divided into two groups. Six were gavaged

Table 1 Haematological results^a from weanling female CD-1 mice dosed with CAPS at levels from 800 to 2000 mg/kg daily for 7 days, and sampled on day 8^b

	0 (Control)	800	Dose level of CAPS (mg/kg)			
			1100	1400	1700	2000
RBC	7.89 (0.30)	8.15 (0.64)	7.72 (0.28)	7.44 (1.00)	7.89 (0.08)	7.06 (0.38)*
HCT	0.446 (0.016)	0.448 (0.031)	0.443 (0.011)	0.413 (0.051)	0.423 (0.010)	0.363 (0.013)***
Hb	13.2 (0.6)	13.5 (1.0)	13.0 (0.6)	12.5 (1.4)	13.1 (0.3)	11.3 (0.2)**
Retic	308.9 (46.2)	257.0 (52.5)	300.3 (94.7)	206.5 (75.1)	6.8 (5.1)***	16.5 (7.3)***
LFR	218.3 (43.6)	183.7 (44.7)	200.0 (66.1)	127.0 (70.0)	6.7 (5.0)**	13.8 (5.7)**
MFR	68.1 (13.1)	49.2 (4.9)	60.6 (31.9)	44.3 (13.7)	0.1 (0.2)**	1.9 (1.5)**
HFR	22.5 (6.3)	24.1 (5.7)	39.7 (17.9)	35.2 (9.7)	0.0 (0.1)**	0.5 (0.4)*
Plt	920 (582)	1229 (161)	1263 (747)	1340 (162)	1171 (234)	1093 (107)
WBC	1.4 (0.5)	1.4 (0.4)	1.8 (0.2)	1.4 (0.2)	1.0 (0.4)	1.4 (1.1)
Myel	29.8 (20.5)	41.6 (5.1)	40.4 (9.8)	47.8 (4.7)	62.5 (3.6)*	61.5 (4.6)*
Eryth	22.4 (15.0)	24.9 (10.6)	34.6 (9.0)	25.8 (7.3)	4.8 (4.0)*	7.5 (6.2)
M:E	1.00 (0.70)	2.08 (1.41)	1.28 (0.58)	1.99 (0.72)	28.19 (30.85)	13.59 (11.32)
FNCC	1.17 (0.14)	1.30 (0.18)	1.10 (0.17)	0.80 (0.66)	0.71 (0.59)	0.99 (0.21)
B. weight	4.4 (1.8)	3.9 (1.6)	4.0 (1.8)	2.8 (0.8)	1.8 (0.8)	1.2 (1.6)

^aValues are means, s.d. in parentheses; $n=3$ at 0, 1400, 1700, 2000 mg/kg, and $n=4$ at 800 and 1100 mg/kg. *Significantly different to control animals, $P<0.05$; ** $P<0.01$; *** $P<0.001$. ^bAbbreviations and units: RBC, red blood cells, $\times 10^{12}/l$; HCT, haematocrit, l/l; Hb, haemoglobin, g/dl; Retic, absolute reticulocyte count, $\times 10^9/l$; LFR, MFR, HFR, low, mid and high fluorescence reticulocytes, $\times 10^9/l$; Plt, platelets, $\times 10^9/l$; WBC, white blood cells, $\times 10^9/l$; Myel, myeloid cells, %; Eryth, erythroid cells, %; M:E, ratio of myeloid: erythroid cells; FNCC, femoral marrow nucleated cell count, $\times 10^7$; B. weight, body weight increase day 1 to day 8, g

Materials and methods

Mouse studies

Animals Female, weanling CD-1 mice and female TO mice (Harlan UK Ltd, Shaw's Farm, Bicester, Oxon, UK), and female, weanling BALB/c mice (A. Tuck and Son Ltd, Beeches Road, Battlesbridge, Essex, UK), were housed in groups of 3–12 on wood shavings with diet (Rat and Mouse No. 1, SDS Ltd, Witham, Essex, UK) and mains drinking water *ad libitum*. A temperature of 19–22°C was maintained, with a relative humidity of 45–65% and a light:dark cycle of 12:12 h (lights on at 07.00 h). Animals were acclimatised for at least 4 days before each experiment and were observed daily for signs of ill health. Body weights were determined daily, or at appropriate times. All animal procedures followed the Home Office (1989) 'Code of Practice for the Housing and Care of Animals Used in Scientific Procedures'.

Administration of chloramphenicol Solutions of chloramphenicol succinate (CAPS; Sigma Chemical Co Ltd, Poole, Dorset, UK) in distilled water were administered to mice by gavage at a constant dose volume of 10 ml/kg body weight; control animals were given water at the same dose volume.

Haematological measurements Animals were killed by i.p. injection of pentobarbitone sodium (Sagatal, Rhône Mérieux Ltd, Harlow, Essex, UK) and blood removed from the right ventricle following a thoracotomy incision. 0.5 ml of blood was anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacriston, Durham, UK). The contents of the right femur were aspirated into 2.0 ml of phosphate buffered saline (PBS) to prepare a marrow cell suspension; a marrow smear was prepared from the contents of the left femur. The sternum was removed and placed in 10.5% phosphate buffered formalin fixative, and sections prepared and stained with haematoxylin and eosin for histological examination.

Blood samples and bone marrow suspensions were analysed with a Technicon H*1 haematology analyser with mouse-specific software (Bayer Diagnostics UK Ltd, Newbury, Bucks, UK), as described previously.^{14–16} For the femoral marrow suspensions, the total nucleated cell count was obtained from the basophil channel of the H*1. Reticulocyte analysis was performed with a Sysmex R-1000 (Toa Medical Electronics, Milton Keynes, Bucks, UK) with voltage gain adjusted optimally for the mouse or rat. Three equal divisions of the total number of reticulocytes gave the percentage low (L), mid (M) and high fluorescence ratio reticulocytes (HFR). Marrow smears were stained with May-Grünwald-Giemsa

and differential counts performed by eye on 200 cells to give the myeloid:erythroid (M:E) ratio.

Serum clinical chemistry Blood was collected into separator tubes (Microtainer, Becton Dickinson and Co, Franklin Lakes, New Jersey, USA), allowed to stand for 75–90 min, centrifuged (6000 r.p.m., 5 min), the serum harvested and stored at approximately –20°C. The following parameters were measured at 37°C using commercial assay kits on a Hitachi 917 Random Discrete Analyser (Boehringer Mannheim, Lewes, E Sussex, UK): alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST), albumin (ALB), total protein (TP), total bilirubin (T-Bil), and total bile acids (BA).

Apoptosis of mononuclear marrow cells Femoral marrow was aspirated into 2.0 ml PBS and the sample placed on ice. Five ml of 1% (w/v) paraformaldehyde in PBS was added, and the mixture placed on ice (15 min) and centrifuged (300 × g, 5 min). The pellet was washed and centrifuged in 5 ml PBS. The cells were resuspended in 0.5 ml PBS and added to 5 ml ice-cold 70% ethanol and stored at –20°C for 24 h. Cells were centrifuged (100 × g, 5 min), resuspended in 1 ml PBS, and centrifuged (twice). The cells were stained in 50 µl fresh staining solution (10 µl TdT reaction buffer [Pharmingen, Cambridge Bioscience, Newmarket Rd, Cambridge, UK], 0.75 µl TdT enzyme [Pharmingen], 8 µl FITC-dUTP [Pharmingen], 32 µl distilled water) at 37°C for 60 min. The staining solution was removed by adding 1 ml PBS and centrifuged (100 × g, 5 min), twice. The cell pellet was resuspended in 1 ml of P1/RNase solution (Pharmingen), incubated at room temperature for 30 min in the dark, and the cells analysed by flow cytometry (FACScan, Becton Dickinson).

Statistical analysis Treated and control groups were compared using *t*-tests for unpaired samples, or Dunnett's test.

Experimental design

Experiment 1 Dose response study CD-1 mice (*n*=3 or 4) were gavaged daily with water or CAPS at 800, 1100, 1400, 1700 and 2000 mg/kg for 7 days and sampled on day 8. Haematological studies were carried out.

Experiment 2 Reversibility of haematological changes CD-1 mice (*n*=7 or 8) were dosed with water or CAPS at 1400 mg/kg, daily, for 10 days and killed at 1, 4 and 15 days after the last CAPS dose. Haematological investigations, and studies on apoptosis and serum clinical chemistry, were carried out.

with water and six with CAPS at 4000 mg/kg. At 20 h post dosing, femoral marrow samples and serum samples were prepared. Femoral marrow nucleated cells were counted, the number of apoptotic mononuclear cells determined, and serum clinical chemistry parameters measured.

Results

Mouse

Experiment 1 Dose response study In mice dosed with CAPS at dose levels from 800–2000 mg/kg for 7 days, mean values for RBC, HCT and Hb were significantly reduced at 2000 mg/kg, and counts for total reticulocytes, LFR, MFR and HFR were significantly reduced at 1700 and 2000 mg/kg (Table 1). Platelet and WBC counts were not reduced by CAPS treatment. Romanowsky stained bone marrow smears from control mice showed a normal distribution of haemopoietic cells with a mean M:E ratio of 1.00:1. In mice treated with CAPS at 1700 and 2000 mg/kg, lymphoid and myeloid cells predominated and erythroid precursors were markedly reduced (Table 1). The M:E ratios were increased at the two highest CAPS dose levels, and ratios of 14.6:1, 26.2:1 and 63.5:1 from individual animals were recorded; however, these changes in M:E ratios were not statistically significant. Cytoplasmic and nuclear vacuolation of erythroid precursors, and of cells of the granulocytic series, was not observed in marrow smears of CAPS-dosed mice. The appearances of marrow smears from control and CAPS-treated mice are shown in Figures 1 and 2. In mice dosed at 1700 and 2000 mg CAPS/kg, sections of sternal marrow showed decreased cellularity within all marrow compartments. This change was due to the loss of erythroid cells and in the more severely affected animals very few erythroid cells were present

(Figures 3–6). As in the marrow smears, cytoplasmic and nuclear vacuoles were not seen in the erythroid and myeloid cells of the sternal marrow of CAPS-treated animals. The femoral nucleated cell counts were not significantly affected by CAPS administration (Table 1). Body weight increases of mice showed a trend of reduction (NS) at the higher CAPS dose levels (Table 1), but the animals showed no clinical signs of CAPS toxicity.

Experiment 2 Reversibility of haematological changes Mice were dosed with CAPS at 1400 mg/kg for 10 days and sampled at 1, 4 and 15 days after the final dose. The administration of CAPS did not affect body weight gain; the mean body weight of control mice at 1 day after the final (vehicle) dose was 23.2 g, whereas CAPS-treated mice weighed 23.8 g. Haematology data are presented in Table 2. RBC, HCT and Hb were significantly reduced in CAPS-treated mice at 1 day after the final dose. However, at day 4,

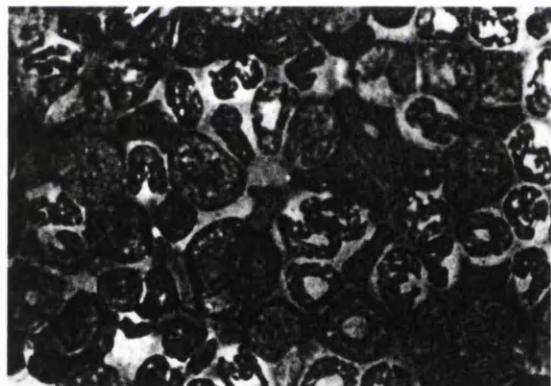


Figure 2 Romanowsky stained bone marrow smear from a mouse treated with CAPS at 1700 mg/kg for 7 days. Erythroid precursors are markedly reduced. The majority of cells present are granulocytic (myeloid), and all stages of development are evident from promyelocyte to segmented neutrophil. A few lymphoid cells are present, but an occasional late normoblast is the only representative of the erythroid series. The M:E ratio is 14.56:1. Magnification $\times 960$

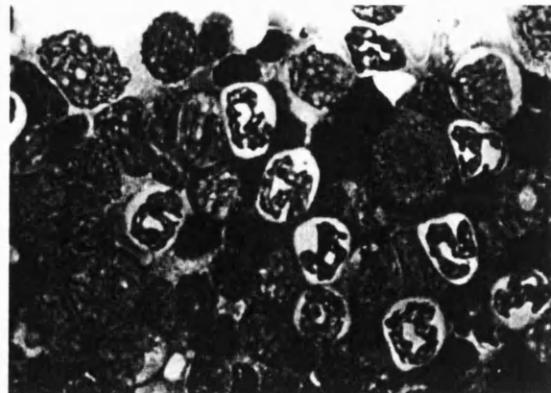


Figure 1 Romanowsky stained bone marrow smear from a control mouse showing a normal distribution of haemopoietic cells; the M:E ratio is 1.08:1. Magnification $\times 960$

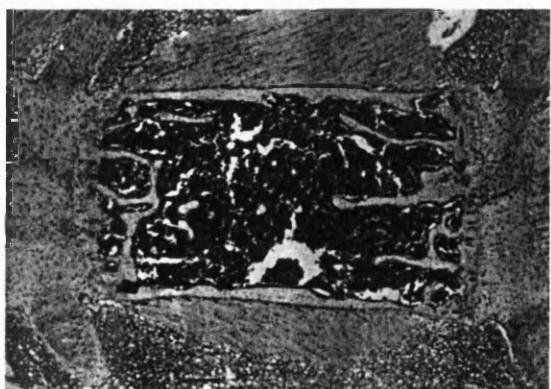


Figure 3 Sternal marrow from a control mouse showing normal cellularity. H&E; magnification $\times 60$

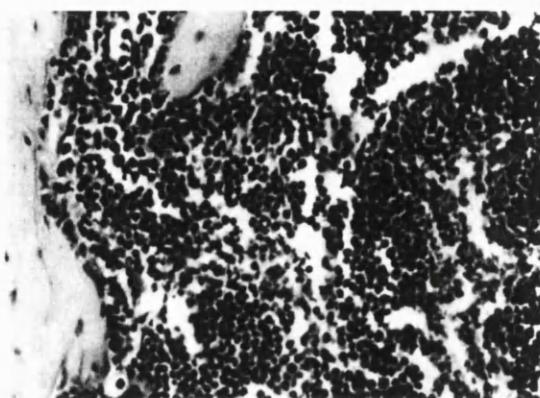


Figure 4 Sternal marrow from a control mouse showing a normal distribution of megakaryocytes, myeloid and erythroid precursors. H&E; magnification $\times 460$

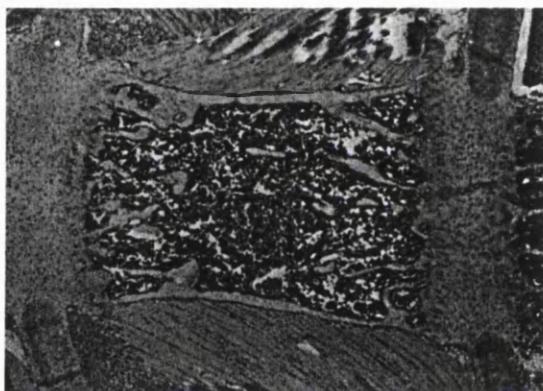


Figure 5 Sternal marrow from a mouse dosed with CAPS at 2000 mg/kg for 7 days; cellularity is moderately reduced. H&E; magnification $\times 60$

parameters were returning towards normal. At day 15, RBC, HCT and Hb values in CAPS-treated mice were higher than in the controls, and this increase achieved statistical significance ($P < 0.05$) in the case of the HCT result. The mean reticulocyte count in CAPS-treated mice was normal at day 1, as were L, M and HFR values. However, at day 4 post dosing, these counts were significantly increased above control values (an 'overshoot response'), but the counts returned to normal by day 15. Platelet counts, and femoral nucleated cell counts, were normal at day one in CAPS-dosed mice, but both parameters were slightly increased above the control value at day 15. Some white blood cell counts were also increased at day 4 and day 15 post dosing, appearing possibly to parallel the 'over-

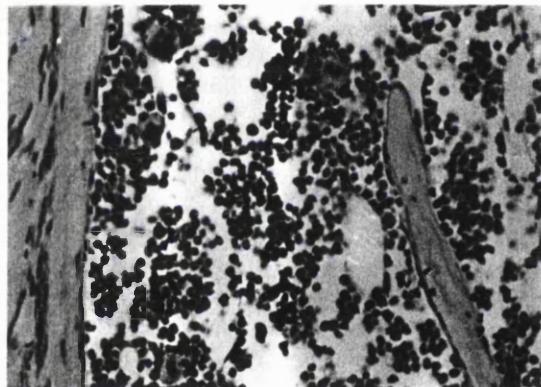


Figure 6 Depletion of erythroid precursors and increased numbers of megakaryocytes in the sternal marrow of a mouse treated with CAPS at 2000 mg/kg for 7 days. H&E; magnification $\times 460$

Table 2 Haematological results^a from weanling female CD-1 mice treated with CAPS daily at 1400 mg/kg for 10 days and sampled at 1, 4 and 15 days after the final dose^b

	Control	Day of sampling			Control	CAPS
		1	CAPS	4		
RBC	8.94 (0.35)	8.38 (0.63)*	8.86 (0.48)	8.66 (0.28)	8.36 (0.64)	8.89 (0.63)
HCT	0.501 (0.017)	0.472 (0.022)**	0.504 (0.020)	0.495 (0.015)	0.469 (0.030)	0.495 (0.025)*
Hb	15.1 (0.4)	14.3 (0.8)*	14.9 (0.5)	14.5 (0.4)	14.2 (0.9)	14.9 (0.7)
Retic	252.5 (60.5)	233.1 (30.3)	163.6 (37.5)	306.6 (35.9)***	277.4 (68.5)	312.8 (38.7)
LFR	148.3 (31.7)	135.9 (22.1)	96.8 (23.1)	162.9 (25.2)***	148.9 (41.9)	169.2 (18.6)
MFR	69.0 (21.6)	59.0 (6.7)	45.8 (10.9)	91.1 (8.7)***	82.7 (22.0)	95.4 (17.9)
HFR	35.1 (13.1)	38.1 (11.6)	21.0 (9.1)	52.6 (17.8)*	45.7 (12.3)	48.2 (18.8)
Plt	989 (105)	1045 (78)	1000 (138)	1090 (108)	944 (207)	1105 (110)*
WBC	1.3 (0.6)	1.4 (0.4)	1.2 (0.5)	2.3 (0.6)**	1.3 (0.5)	2.6 (1.5)*
Neut	0.64 (0.1)	0.39 (0.17)	0.41 (0.10)	0.70 (0.22)**	0.35 (0.10)	0.54 (0.24)*
Lymph	0.84 (0.51)	0.85 (0.21)	0.66 (0.33)	1.44 (0.41)***	0.86 (0.41)	1.87 (1.31)*
Mono	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.01 (0.02)***	0.05 (0.02)	0.07 (0.02)
LUC	0.03 (0.02)	0.03 (0.01)	0.02 (0.01)	0.05 (0.03)*	0.01 (0.01)	0.03 (0.02)
Myel	37.8 (7.5)	36.0 (2.7)	—	38.1 (4.6)	—	35.9 (3.2)
Eryth	29.2 (3.7)	34.9 (4.5)*	—	33.6 (5.1)	—	33.5 (3.1)*
M:E	1.34 (0.49)	1.05 (0.16)	—	1.17 (0.33)	—	1.08 (0.17)
FNCC	1.40 (0.27)	1.24 (0.38)	1.44 (0.25)	1.34 (0.31)	0.96 (0.38)	1.30 (0.16)*

^aValues are means, SD in parentheses; $n=8$, except 1400 mg/kg groups at day 4 and 15 where $n=7$, and results for Myel, Eryth and M:E where $n=5$. *Significantly different to control animals at same time point, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; but for Myel, Eryth and M:E, all CAPS-treated mouse groups were compared with control animals at day one. ^bAbbreviations and units as Table 1, except: Neut, neutrophils, $\times 10^9/l$; Lymph, lymphocytes, $\times 10^9/l$; Mono, monocytes, $\times 10^9/l$; LUC, large unstained cells, $\times 10^9/l$.

'shoot response' in the reticulocyte and platelet counts.

Bone marrow smears from some animals (control mice at day 1, and CAPS-treated mice at day 1, 4 and 15) were examined to assess changes in M:E ratios (Table 2). CAPS treatment at 1400 mg/kg did not affect the M:E ratio at day 1 post dosing; similarly, the percentage of myeloid cells was not affected by treatment with the antibiotic at this time. However, the percentage of erythroid cells was slightly but significantly increased at day 1 and day 15.

The number of apoptotic mononuclear cells in femoral marrow samples was assessed in control and CAPS-treated mice at day 1, 4 and 15 after dosing. The results are presented in Table 3. The absolute number of apoptotic cells was increased in CAPS-dosed mice at each time point, and the values achieved statistical significance at day 1 and 15 post dosing.

Experiment 3 Apoptosis and serum clinical chemistry study Mice were given a single dose of water (control), or CAPS at 2200 mg/kg, and sampled at 18 h; results are set out in Table 4. The number of apoptotic femoral mononuclear cells was significantly increased in CAPS-treated mice; serum clinical chemistry analysis demonstrated slight increases in AST and ALT activities, and a minor decrease in TP.

Experiment 4 CAPS acute toxicity studies In an initial experiment, weanling BALB/c mice given a single gavage dose of CAPS at levels from 3000–5500 mg/kg showed no clinical evidence of adverse effects; at 6000 mg/kg of CAPS, and above, the animals demonstrated temporary signs of toxicity. Those treated at 6000 mg CAPS/kg recovered, while those given 6500 and 7000 mg/kg were killed, it being considered that the toxic changes were not reversible. In a second study, TO mice (32 g; n=4) were given a single dose of CAPS from 3500–7500 mg/kg. One mouse in each of the 5000, 7000 and 7500 mg/kg dose groups showed slight signs of toxicity within 1 to 2 h of drug administration but the effects did not become more severe over the following 2 h, and it

was considered that the animals were likely to recover. However, each of these three mice was found dead the following day at the early morning inspection. No other CAPS-treated animals showed any clinical signs of toxicity at any of the dose levels tested.

Rat

Experiment 1 Dose response study Control rats increased in mean body weight from 131.0 g at the beginning of the study to 239.0 g at the end, an increase of 108.0 g. At 2000–3200 mg/kg CAPS, there were no significant effects on body weight gain, but at 3600 and 4000 mg CAPS/kg the mean body weight increase during the study was reduced to 89.0 g and 91.3 g, respectively; these (reduced) increases in body weight gain were significantly lower than in the controls ($P<0.05$ and $P<0.001$, respectively).

There were no clinical signs of CAPS-induced toxicity in rats given the drug at any dose level. However, at the post mortem examination, at all dose levels, the caecum and colon, and to a lesser extent the small intestine and stomach, were swollen and slightly gas filled, and on occasion

Table 4 Results^a from apoptosis in mononuclear femoral marrow cells and serum biochemistry analysis in CD-1 mice given a single oral dose of CAPS at 2200 mg/kg and sampled at 18 h^b

	Control (vehicle)	CAPS
Apoptotic cells ($\times 10^7/l$)	9.5 (1.8)	15.8 (7.6)*
Apoptotic cells (%)	9.13 (1.61)	14.79 (5.73)*
FNCC (10^7)	1.07 (0.23)	1.06 (0.23)
ALP (IU/l)	487.8 (99.2)	428.7 (144.6)
ALT (IU/l)	29.2 (2.6)	38.3 (5.9)**
AST (IU/l)	102.9 (26.4)	127.0 (32.1)
ALB (g/l)	27.3 (1.3)	26.7 (1.1)
TP (g/l)	47.0 (1.8)	43.6 (3.1)*
T-Bil ($\mu\text{mol/l}$)	1.60 (0.39)	1.53 (0.42)
BA ($\mu\text{mol/l}$)	18.78 (1.60)†	18.68 (4.48)

^aValues are means, s.d. in parentheses; n=6, except † where n=5.

^bSignificantly different to control animals, $P<0.05$; ** $P<0.01$.

Abbreviations: FNCC, femoral nucleated cell count; ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; ALB, albumin; TP, total protein; T-Bil, bilirubin; BA, bile acids

Table 3 Analysis of apoptotic mononuclear cells in femoral marrow samples of CD-1 mice dosed daily with CAPS at 1400 mg/kg for 10 days, and sampled at days 1, 4 and 15 after the final dose^a

	Day of sampling		Day of sampling		Day of sampling	
	1	CAPS	4	CAPS	15	CAPS
Control	Control	Control	CAPS	Control	CAPS	
Apoptotic cells ($\times 10^7/l$)	22.8 (14.7)	57.0 (22.2)**	26.0 (9.2)	47.0 (36.2)	13.5 (6.8)	34.3 (16.4)**
Apoptotic cells (%)	28.42 (16.77)	37.68 (10.23)	21.81 (7.12)	23.74 (6.87)	10.63 (0.85)	12.90 (1.78)*

^aValues are means, s.d. in parentheses; n=5 except control data at day 1 and 4, where n=4. *Significantly different to control animals at the same time point, $P<0.05$; ** $P<0.01$.

the gut contents were pale in colour. However, faecal pellets were of normal colour and consistency.

Haematological results, in general, showed no effect of CAPS administration on leucocyte parameters, or on platelet counts (Table 5). However, Hb values demonstrated a clear dose related decrease at higher CAPS dose levels and this change was significant at 3600 ($P<0.05$) and 4000 mg/kg ($P<0.01$); significant effects were not evident in the results for RBC and HCT. Mean cell volume (MCV) values were unaffected by CAPS administration. Mean cell haemoglobin (MCH) values were slightly reduced at 3200, 3600 and 4000 mg/kg, and the reduction was significant at the 3600 mg/kg dose level ($P<0.05$). Mean cell haemoglobin concentration (MCHC) values were significantly lower at the 3200, 3600 and 4000 mg/kg dose levels ($P<0.001$, $P<0.001$, $P<0.01$, respectively). Total reticulocyte counts, and LFR and HFR counts, were not affected by CAPS dosing; however, although generally the MFR values were also not changed, the count at 2800 mg/kg was slightly but significantly lower than the controls ($P<0.05$). Microscopic examination of Romanowsky stained marrow smears demonstrated no effects of CAPS administration, and no cytoplasmic or nuclear vacuolation of erythroid or other cells was evident in drug-treated animals.

The administration of CAPS at levels of 2000–4000 mg/kg did not alter the values of coagulation screening tests (Thrombotest, PT and APTT). However, values for fibrinogen were increased above the controls at all CAPS dose rates, and these

effects were statistically significant at levels from 2000–3600 mg/kg (Table 5).

Experiment 2 Apoptosis and serum clinical chemistry study The administration of CAPS at 4000 mg/kg to Wistar Hanover rats did not affect the total number of femoral marrow nucleated cells: mean, control, 4.17; CAPS-treated, 4.15×10^7 . The absolute number of apoptotic mononuclear cells in femoral samples was also not significantly affected by CAPS administration: mean, control, 0.250; CAPS-treated, 0.477×10^6 ; these counts were 0.58 and 1.17%, respectively, of the total number of mononuclear cells.

Analysis of serum clinical chemistry parameters in control and CAPS-treated rats illustrated few changes. There was a significant decrease in CAPS-dosed animals in the mean ALT value (control, 57.33; CAPS, 47.23 IU/l; $P<0.05$), a decrease in mean urea (control, 9.11; CAPS, 6.77 mmol/l; $P<0.01$), and a decrease in the mean cholesterol level (control, 1.638; CAPS, 1.093 mmol/l; $P<0.05$).

Discussion

There are few reports on the *in vivo* haemotoxicity of chloramphenicol in small laboratory animals, and there are no studies investigating the chloramphenicol-induced reversible anaemia seen in man. However, some attempts to induce AA in laboratory rodents using chloramphenicol have been described.^{18,23–25} The induction of neoplasia (lymphoma) by chloramphenicol was described by

Table 5 Haematological results^a from male Wistar Hanover rats dosed with CAPS at levels from 2000–4000 mg/kg daily for 19 days, and sampled on day 20^b

0 (Control)	2000	2400	Dose level of CAPS (mg/kg)			
			2800	3200	3600	4000
RBC	6.87	7.00	6.65	6.72	6.81	6.76
HCT	0.414	0.418	0.397	0.402	0.416	0.412
Hb	13.5	13.6	13.1	13.1	12.7	12.5*
MCV	60.2	59.7	59.7	59.9	61.3	60.9
MCH	19.7	19.4	19.7	19.5	18.7	18.5*
MCHC	32.7	32.4	33.0	32.6	30.5***	30.4***
Retic	292.0	297.8	287.7	262.7	280.5	277.6
LFR	120.5	120.2	121.7	114.4	116.6	110.6
MFR	103.2	102.0	85.8	84.9*	91.3	91.5
HFR	68.2	75.7	80.2	63.3	72.7	75.5
Plt	807	908	888	902	941	900
WBC	4.5	4.7	4.6	2.9*	3.8	3.0
Neut	0.58	0.57	0.55	0.52	0.56	0.34***
Lymph	3.68	3.97	3.88	2.26	3.17	2.60
Thrombo	41.7	43.9	43.4	44.5	42.5	41.5
PT	24.0	23.9	24.8	24.1	23.1	22.9
APTT	16.4	16.6	17.6	15.1	14.7	14.4
Fbgn	1.70	1.98*	1.99**	2.00*	1.95*	1.97*
B. weight	108.0	86.0	97.0	91.0	94.0	89.0*
						91.3***

^aValues are means; $n=5$ (controls) or $n=3$ (all CAPS groups). *Significantly different to control animals, $P<0.05$; ** $P<0.01$; *** $P<0.001$. ^bAbbreviations and units as Tables 1 and 2, except: MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Thrombo, Thrombotest, seconds; PT, prothrombin time, seconds; APTT, activated partial thromboplastin time, seconds; Fbgn, fibrinogen, g/l

Robin *et al.*²⁴ however, other workers have not confirmed this result.¹⁵

A feature of several of the earlier haematological investigations with chloramphenicol in the mouse, is that relatively low doses of the drug were used. For example, Robin *et al.* administered an estimated 100 mg/kg of CAPS to mice, 5 days per week for 5 weeks, and reported no haematological changes.²⁴ A reduction in reticulocyte count, but no other effects, was reported by Hara *et al.* with CAPS injected subcutaneously at an estimated dose level of 800 mg/kg for 5 days.²⁶ Vácha *et al.* injected CAPS subcutaneously at 320 mg/kg every 8 h for 5 days; no changes in blood were identified.²⁷ However, in contrast, Firkin *et al.* injected chloramphenicol at 600 mg/kg every 8 h for 5 days; a reduction in reticulocytes was seen, but no other haematological effects.²⁸

In a previous investigation we reported a 13 month experiment,¹⁶ based on work of Morley *et al.*¹⁸ with CAPS administered to mice in the drinking water at 4.0 mg/ml; the consumption of CAPS ranged from 176–370 mg/kg/day. Blood was examined at various time points, but no changes were seen that could be attributed to the antibiotic.

However, some workers have administered higher levels of chloramphenicol to mice. Gruhztit *et al.* reported that the maximum tolerated oral dose of chloramphenicol in the mouse was 1500 mg/kg, and that the acute oral LD₅₀ was 2640 mg/kg.²⁹ Similarly, Fritz and Hess administered 500–2000 mg/kg chloramphenicol orally to mice in repeat dose teratogenicity studies.³⁰ It was therefore concluded that levels of CAPS up to 2000 mg/kg, could be administered to mice in repeat dose studies to examine the haematological response.

In mouse Experiment 1, CAPS induced significant reductions in RBC, HCT and Hb, and decreases in HFR, MFR, LFR and total reticulocyte counts at 2000 mg/kg; effects were dose related. Platelet and WBC counts were unaffected. In Experiment 2, with CAPS dosed at 1400 mg/kg, RBC, HCT and Hb, values were significantly lower at day 1 after dosing, but returned to near normal values by day 15; platelet and WBC counts were not affected at day 1 post dosing (Table 2). When the features of this response are compared with the reversible anaemia seen in man, there are close similarities: the murine response is a mild, dose-related anaemia with reticulocytopenia. It develops during treatment, is reversible, and the changes revert to normal rapidly. The marrow shows increased M:E ratios, and erythroid cells are reduced in number. However, in contrast to the human condition, the response in the mouse was not associated with leucopenia or thrombocytopenia, and the marrow cellularity was decreased.

There are no reports of the haematological response to chloramphenicol in the rat. In rat

Experiment 1, there were dose-related reductions in Hb values (at 3600 and 4000 mg/kg), and MCHC values were also reduced at higher CAPS dose levels (3200–4000 mg/kg); there were no effects on platelet and WBC counts, nor on Thrombotest, PT and APTT values. In summary therefore, the administration of CAPS at 4000 mg/kg for 19 days in the rat did induce a mild anaemia, with a reduction in Hb values to about 91% of the controls.

There are several reports of chloramphenicol-induced cytoplasmic and nuclear vacuolation in the marrow of patients.^{31–33} This change involves mainly the erythroblast (pro-erythroblast), but granulocytes, megakaryocytes and plasma cells were also sometimes affected. Schober *et al.*³⁴ produced this effect in rats injected twice daily with CAPS at 150 mg/kg for 5 weeks; vacuoles were observed in the pronormoblast at the nuclear-cytoplasmic junction, and in myeloblasts and promyelocytes. In the present study however, vacuolation was not evident in the marrow smears of CAPS-treated mice or rats, nor in mouse sternal marrow. In a recent study we have dosed BALB/c mice (n=3) with a single oral dose of CAPS at 3500 mg/kg, or with the related antibiotic thiamphenicol (1750 mg/kg), and examined femoral marrow at 18 h post dosing using transmission electron microscopy, and tibial marrow with light microscopy; no cytoplasmic or nuclear vacuolation was observed in any cell lineage.

In the rat, but not in the mouse, CAPS caused evidence of intestinal disturbance. This may have been associated with a disruption of the normal enteric flora with possible bacterial overgrowth.³⁵ The raised blood fibrinogen levels (Table 5), as an acute phase protein and an indicator of an inflammatory process, may have been associated with this enterocolitis. However, chloramphenicol is a relatively poor inducer of enterocolitis, and the drug tends not to produce significant toxicity when administered therapeutically to small laboratory animals.³⁵

Investigations on coagulation factors were conducted in the CAPS-treated rat. It was considered that the administration of the antibiotic may have induced changes in the intestinal flora with resulting adverse effects on vitamin K metabolism, and levels of vitamin K-dependent-blood coagulation factors. This has been documented for certain penicillins and cephalosporins, with the result that homeostasis is compromised.^{36,37} However, Thrombotest, PT and APTT assays demonstrated that coagulation in CAPS-treated rats was normal, and there was no depletion of vitamin K-dependent coagulation factors. Similarly, daily examination of the animals had demonstrated no external evidence of haemorrhage, and none was grossly evident internally at autopsy. Nevertheless, these negative findings contrast with those of Bengmark *et al.* who

showed increased bleeding times and blood loss in rats given 50 mg/kg of CAPS.³⁸

In early toxicity studies carried out with chloramphenicol (introduced as chloromycetin by Parke-Davis and Co, in 1947–1948), it was reported that the drug administered by the oral route to mice gave a maximum tolerated dose of 1500 mg/kg, and an LD₅₀ of 2640 mg/kg.^{29,39} These early reports, even now, represent the only data available, and are still quoted.⁵ There are no comparable results reported for CAPS. However, in parallel experiments, we had recently examined the haemotoxicity of CAPS in a variety of mouse strains, employing levels up to 2500 mg/kg, and up to 3000 mg/kg in the CD-1 mouse. In none of these studies were clinical signs of CAPS toxicity seen. As these parallel experiments with CAPS appeared to contrast markedly with those of Gruhitz *et al.* using chloramphenicol,²⁹ we examined the single dose oral toxicity of CAPS. Dose levels of the order of 6000 mg/kg were tolerated in the BALB/c mouse and 4500 mg/kg in the TO mouse.

Philpott *et al.* demonstrated increased apoptosis in bone marrow progenitor cells of AA patients.⁴⁰ Holt *et al.* showed that chloramphenicol initiated apoptosis in a kidney-derived cell line, and haematoopoietic progenitor cells from human neonatal cord blood.⁴¹ Evidence was also presented indicating increased apoptosis in bone marrow cells of mice given a single dose of CAPS.¹⁶ We therefore wished to examine apoptosis in marrow mononuclear cells in mice given several daily doses of CAPS (Table 3). Apoptosis was increased at days 1, 4 and 15 post dosing, and levels were significantly higher at days 1 and 15. A similar positive effect was found in mice treated with a single dose of CAPS at 2200 mg/kg (Table 4). However, administration of a single dose of CAPS to rats did not increase the number of apoptotic cells. These data require further investigation. They indicate in the mouse, but not in the rat, that CAPS may initiate apoptosis in mononuclear marrow cells, but that a later-stage effect may also occur, affecting apoptosis in the post-dosing period, as the marrow returns to normal.

Mice given a single dose of CAPS at 2200 mg/kg were examined for changes in serum clinical chemistry (Table 4); the parameters examined were indicators of liver injury. The slight increases in AST and ALT, and the minor decrease in TP, suggest mild hepatic injury. Using a wider set of assays, the changes seen in rats dosed with CAPS at 4000 mg/kg were few, and relatively minor. However, it is considered that the decreases observed in the rat in ALT, urea and cholesterol may be associated with gastro-intestinal disturbance, and an effect on nutrient uptake. There are no reports with which to compare the present findings, except that of Mandal *et al.* who administered chloramphenicol (preparation, vehicle, route not given) at

50 and 100 mg/kg to Wistar rats for 21 days.⁴² Hepatotoxicity was reported, with increased levels of AST and ALT in the liver and serum, and increased ALP in the liver; effects were dose-related. Our results therefore contrast with these findings.

An important question to emerge from a consideration of the dose-related reversible anaemia caused by chloramphenicol in man, is the possible relationship between this condition and chloramphenicol-induced AA. It was originally thought that in man, the reversible form of haemotoxicity could, and possibly would, progress to irreversible aplasia.⁴³ For this reason, the initial stages of the reversible anaemia were thought of as an early warning of a more serious bone marrow depression to come,³¹ or as a reversible stage in the development of irreversible AA.⁴⁴ These ideas were subsequently discussed by Yunis and Bloomberg,¹² who considered that the two types of chloramphenicol haemotoxicity were unrelated to each other. They concluded that, in man, there was no evidence that the continued administration of chloramphenicol would lead to aplasia, nor that the early discontinuation of the drug would prevent the subsequent development of bone marrow aplasia.⁴⁵ Viewed against this background, development of the present mouse model of reversible CAPS-induced anaemia may allow the relationship between the reversible form of anaemia, and AA, to be investigated experimentally. However, the marrow lesion induced in the present studies does not have the characteristics of stem cell injury (as would be present in progress toward AA), but appears to be an effect on later, committed progenitor cells of the erythroid lineage. Nevertheless, we have recently examined whether the reversible CAPS-induced anaemia in the mouse will develop into a later-stage AA; these studies will be reported in the near future.

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Complete Automated Evaluation of Haematological Parameters Using the Technicon H*1 Haematology System: Experimental Studies with Busulphan in the Mouse

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Aplastic anaemia (AA) is characterized by peripheral blood pancytopenia and bone marrow hypocellularity, and is often seen as an idiosyncratic reaction to certain drugs (e.g. chloramphenicol, phenylbutazone, sulphonamides). No practical system exists for screening drugs for the induction of idiosyncratic AA, but Morley *et al.*¹ proposed a mouse model which involved the initial sensitization of haemopoietic stem cells with busulphan, followed by chloramphenicol treatment. However, Morley did not validate this proposed model. We wish to develop techniques to assess the potential of drugs to induce AA and have begun these studies by investigating the effects of busulphan on mouse peripheral blood using the Technicon H*1 analyser.

The H*1 (Bayer UK Ltd) is an automated haematology analyser employing flow cytometry to produce a large number of measurements from a small volume (100 μ l) of blood.² Recently, software has become available allowing the H*1 to be used in laboratory animal haematology. As well as a screen of 'standard haematology parameters' routinely assessed in toxicological studies, several 'non-standard parameters' are also produced by the H*1. Using this analyser we wished to determine i) the relative sensitivity of erythrocytes, neutrophils, lymphocytes, monocytes and platelets to busulphan ii) whether the effects are dose related iii) the usefulness of the 'non-standard parameters' produced by the H*1.

Forty-eight female B6C3F1 mice (10-weeks-old) were dosed ip on four occasions at 14-d intervals with busulphan (Sigma) at 0, 10, 15, 20, 25, 30, 35 and 40 mg kg⁻¹, six mice per group. Fourteen days after the final dose, mice were autopsied after CO₂ overdose and 0.5 ml blood added to K₂ EDTA; 100 μ l aliquots were analysed using the H*1. Automated counting of reticulocytes was carried out using a Sysmex R-1000.

The administration of busulphan induced dose-related effects on a wide range of blood parameters. In treated mice, changes (↑, increase; ↓, decrease) in erythrocyte count (RBC, ↓), mean cell volume (MCV, ↑), mean cell haemoglobin (MCH, ↑), platelet count (PLT, ↓) and monocyte count (MONO, ↓) were statistically significant ($P<0.001$). However, changes in neutrophil count (NEUT, ↓) and lymphocyte count (LYMPH, ↓) were not statistically significant. The 'non-standard parameters' % macrocytic erythrocytes (% MACRO) and % hypochromic erythrocytes (% HYPO) were each significantly increased ($P<0.01$) in busulphan-treated mice. Another 'non-standard parameter', the mean peroxidase index (MPXI), a measure of neutrophil peroxidase staining intensity (an indicator of neutrophil function and age), was increased in treated mice, but not to a significant level. The reticulocyte count (RETIC) was also increased by busulphan administration, but not significantly.

Morley and Blake³ demonstrated a depression of RBC, NEUT, LYMPH and MONO values in busulphan-treated mice. Our data also show a depression of these parameters, and also for PLT, but the decreases for NEUT and LYMPH were not statistically significant. Increases in the 'non-standard parameters' % MACRO and % HYPO were of interest, and probably associated with the raised RETIC counts. We consider the increases in % MACRO and MPXI may indicate an underlying busulphan-induced megaloblastic erythroid development,⁴ which has also been reported in patients undergoing cytotoxic drug treatment⁵ and is related to the failure of DNA repair mechanisms. Our studies, with particular reference to changes in % MACRO, % HYPO, MPXI and RETIC values are continuing, to investigate further the basis of the busulphan-induced lesion.

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Myelotoxicity in Female B6C3F1 Mice Induced by Busulphan

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We have previously examined the induction of hypoplastic anaemia in female B6C3F1 mice using busulphan. In mice given 4 doses of busulphan (10 to 40 mg kg⁻¹) at fortnightly intervals there was persistent anaemia, leucopenia and thrombocytopenia at 2 weeks after the final busulphan dose.¹ Using the Technicon H¹ and specialised software for mouse blood, it was also possible to demonstrate an increase in the mean neutrophil peroxidase index (MPXI) and numbers of macrocytic and

hypochromic erythrocytes. Furthermore, reticulocyte numbers were reduced, but the decrease was not statistically significant. Following this initial study, we have now examined the effect of busulphan on the amplitude of a range of parameters in sequential blood samples taken over a 10 day period. Peripheral blood changes were also linked to measurements of bone marrow and spleen cellularity.

Ninety mice in 3 groups were dosed (day [d.] 0) with vehicle (control) or busulphan (Sigma) at 35 or 45 mg kg⁻¹ i.p. On d.1, 2, 3, 4, 7 and 10, 5 animals per group were killed by CO₂ overdose and 0.5 ml blood taken into K₂EDTA. Marrow from the left femur was flushed into 10 ml saline (Isoton II, Coulter); spleens were removed, scissor-minced and cavitated with a 50 ml syringe and suspended in 10 ml Isoton. Blood, marrow and spleen samples were analysed by H¹; reticulocytes were counted using a Sysmex R-1000 analyser.

In peripheral blood samples, results over the 10 day period, at 35 and 45 mg kg⁻¹ of busulphan, showed dose dependent reductions in the counts of all leucocyte types, but generally the results were not statistically significant. Neutrophil MPXI was increased in busulphan-treated mice; circulating platelet numbers were not affected. At 45 mg kg⁻¹, erythrocyte counts (RBC) began to fall at d.4, with a statistically significant decrease at d.7, and a partial recovery at d.10. A similar pattern of RBC change occurred at 35 mg kg⁻¹ but the decreases were not statistically significant. These effects on RBC were paralleled by similar patterns of change, with significant decreases, in

nucleated cell counts from spleen and marrow, but these reductions occurred 48 h before those seen in peripheral RBC. R-1000 analysis showed total reticulocyte counts, and the numbers of low (LFR), middle (MFR) and high (HFR) fluorescence fractions, paralleling the time course and patterns of change seen in the femur and spleen nucleated cell counts. Calculation of reticulocyte Maturation Index² [MI = ((MFR+HFR)/LFR) × 100%] produced plots similar in pattern and time course to the total reticulocyte counts and to the LFR, MFR and HFR fractions. However, in addition, the MI clearly demonstrated a rebound reticulocytosis at d.7 (45 mg kg⁻¹) and d.4 (35 mg kg⁻¹).

We therefore conclude that the profound short term bone marrow changes in this model of busulphan-induced myelotoxicity in female B6C3F1 mice can be precisely and sensitively characterised by simple peripheral blood measurements.

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Abstracts

Association for Comparative Haematology Autumn Meeting 1995

The Autumn 1995 Meeting of the Association for Comparative Haematology took place on 9th and 10th November at Norwich. It was sponsored jointly by Bayer Diagnostics and Organon-Teknika UK, and was organised by Pharmaco-LSR (now part of Huntingdon Life Sciences Ltd). Thursday afternoon, 9 November, was devoted to a workshop on Reticulocyte Counting, where a free exchange of views/information took place following a series of short introductory talks. The Friday programme was more general, with topics covering toxic effects of experimental compounds, problems of high APTT's in dogs, and *In Utero* Transplantation of Haematopoietic Cells.

The next Meeting of the Association is scheduled for April 1996 at Welynn, UK.

Sensitivity of Reticulocyte High Fluorescence Ratio to Splenic Erythropoiesis C. M. Andrews, Glaxo Wellcome Research & Development

Mice dosed intraperitoneally with between 5–100 mg methotrexate per kg showed a dose-related decrease in High Fluorescence Ratio (HFR) reticulocytes three days post dose as measured by the Sysmex R-1000. HFR returned to pretreatment levels in animals dosed with 5–20 mg/kg, but at 50 and 100 mg/kg, there was a rebound effect at day 8 which had returned to control levels by day 15. Bone marrow erythroid counts (from combined H*1 basophil channel count and visual M:E ratio) for the 100 mg/kg group show no such rebound at day 8.

H & E stained spleen sections were graded visually by an (inexperienced) observer for erythropoietic activity: 0 = no erythroid cells, 1 = few (normal) to a maximum of 5. This microscopic assessment was performed 'blind'. Control spleens were generally graded 1 whilst the 100 mg/kg group dropped to 0 on day 3, ranged from 2 to 5 on day 8 and from 0 to 1 on day 15. When plotted against each other, HFR and splenic erythropoietic grade showed fair correlation ($r = 0.67$).

HFR may be used as a measure of bone marrow engraftment in man (Davis et al. 1992) and myelotoxicity in mice (Andrews et al. 1993). It would appear that it may also accurately mirror splenic erythropoiesis in the absence of changes in the bone marrow. The spleen has long been known to be of importance in rodent erythropoiesis (Bozzini et al. 1970): measurement of HFR represents a useful (non-terminal, in the case of mice) means of charting splenic erythropoietic activity. Further work on this project is being carried out, and will be reported in full at a later date.

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Chloramphenicol-induced reticulocytopenia and anaemia in the mouse: depressed parameters return to normal with continued dosing

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Chloramphenicol is haemotoxic in man, inducing two effects. First, aplastic anaemia, evident in blood as pancytopenia, is often irreversible and fatal. Second, a mild anaemia with reticulocytopenia; this is dose-related, develops during treatment, and is reversible. We have developed a model of the dose-related anaemia which shows many features of the human condition, 'in *essence*: chloramphenicol succinate (CAPS) is administered to CD-1 mice by gavage at 1400 mg/kg daily, for 10 days. However, in a recent study we administered CAPS daily, at 2000 mg/kg, to BALB/c mice for 17 days. Surprisingly, it appeared that after 17 daily doses, the severity of the anaemia, and reticulocytopenia, had not increased, and haemoglobin (Hb), erythrocyte and reticulocyte values were returning towards normal. We have therefore investigated this apparent effect, with CAPS administered at 2 levels for 20 days.

Female BALB/c mice were gavaged with water (control) or CAPS at (250 and 2500) mg/kg daily. Control mice were sampled at day (d) 1 (first day of CAPS dosing), and from control and CAPS groups at d6, 12, 16 and 21 (i.e. after 5, 11, 15 and 20 doses); n = 4-6. Blood was taken and analysed.¹ Results for Hb, as an example of erythrocyte changes, and reticulocytes, are presented (Table). At 1250 mg/kg, Hb values were significantly reduced at d6, but were returning to normal at d12, and were comparable to controls at d16 and 21. Reticulocytes were significantly reduced at d6, but normal at d12, and slightly higher than controls at d16 and 21 (NS). Platelets were higher than controls at each time point (NS). At 2500 mg/kg, Hb was significantly reduced at d6, and this was more pronounced at d12; at d16 and 21, Hb was lower than controls, but returning towards normal. Reticulocytes were reduced at d6, returning towards normal at d12 and 16, and were significantly higher than controls at d21 ("overshoot" response). Platelets were higher than controls at all times.

Therefore, the response in CAPS-dosed mice was an early reduction in Hb and reticulocytes, with a return towards normal as drug administration continued, platelets were consistently higher than controls. Apparent "tolerance" to CAPS toxicity may involve the drug disturbing its own metabolism with non-formation of toxic metabolites; e.g. UDP-glucuronyl transferase induction would increase formation of easily excretable glucuronide conjugates, decreasing CAP availability for metabolism to haemotoxic aldehyde or nitroso derivatives. Raised platelets are possibly a response to increased erythropoietin (EPO) levels, resulting from the initial anaemia (EPO stimulates erythroid and megakaryocyte lineages).

Table: Mean haematological results from BALB/c mice dosed with CAPS daily for 5, 11, 15 and 20 days at 1250 and 2500 mg/kg, and sampled at day 6, 12, 16 and 21, respectively

	CAPS dose (mg/kg)	Day of sampling				
		1†	6	12	16	21
Reticulocytes ($\times 10^6/\text{dL}$)	Control	354	399	403	281	277
	1250		10***	287	469	329
	2500		11***	80	99	801*
Hb (g/dL)	Control	14.7	14.7	14.4	15.0	15.5
	1250		13.5*	13.8**	14.9	15.2**
	2500		13.4***	12.4***	13.0*	13.6***

*Control mice were also sampled on day 1 of CAPS dosing. †significantly different to controls, p<0.05; *p<0.01; **p<0.001.

¹Yallop, D., Andrews, M., Holt, D. et al. (1998) *Hum. Exp. Toxicol.* 17, 57.

Robinson, S., Hayard, A., Andrews, M. et al. (1999) *Hum. Exp. Toxicol.* in press.

²Holt, D., Andrews, M., Payne, J. et al. (1998) *Hum. Exp. Toxicol.* 17, 8-17.

Chloramphenicol induces reversible dose-related reticulocytopenia, not aplastic anaemia, in the guinea pig

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In man, chloramphenicol (CAP) induces, first, a dose-related, reversible reticulocytopenia and anaemia, developing during treatment; second, a non-dose-related, aplastic anaemia (AA) which becomes evident weeks after treatment and is often fatal. We have attempted to induce AA in the mouse with CAP but have been unsuccessful.¹ The literature has revealed no reports of CAP inducing AA in laboratory animals, except a single account in the cat.² As CAP is haemotoxic in the rat only at high levels,³ we examined the haematological response in another rodent. We have investigated whether CAP induces AA in the guinea pig.

Weanling female Dunkin Hartley guinea pigs were gavage dosed daily with CAP succinate (CAPS) in water in 3 preliminary dose ranging studies. Doses of 1000, 2000, 3000 and 4000 mg/kg were given for 9 days (Study 1), 2500 and 3500 mg/kg for 9 days (Study 2), and 333, 666 and 1000 mg/kg for 13 days (Study 3); n= 2 to 5. Haematological studies were carried out on the day after the final CAPS dose, as described.³ In Study 4, guinea pigs were treated with water (n=3) or CAPS (n=4 or 5) at 825 mg/kg for 16 days and examined at day (d) 1, 12, 28 and 63 after the final CAPS dose.

In the 3 preliminary studies a pattern of haematological changes occurred, with significant CAPS-induced reticulocytopenia, and a reduction in femur (femoral) marrow cell count (FMCC). However, a haemocencentration effect was also apparent, with increased erythrocyte (RBC) counts and haemoglobin (Hb) values. Results in Study 4 are given (Table). Reticulocytes were significantly reduced at d1, with an "overshoot" at d12, returning to normal at d28; FMCC was decreased at d1, but normal thereafter; Hb, platelets and white blood cells were normal at d1. At d63 all blood values were normal in CAPS-treated animals.

We conclude therefore that in the guinea pig, as in man, CAPS induces a reversible, dose-related reticulocytopenia, which develops during treatment. There was no evidence of a "late stage" (d63) reduction in blood values, as would be seen in an overt or developing bone marrow aplasia.

Table: Mean haematological results from Dunkin Hartley guinea pigs dosed with CAPS daily for 16 days at 825 mg/kg, and sampled at day 1, 12, 28 and 63

Day of sampling	1		12		28		63	
	Control	CAPS	Control	CAPS	Control	CAPS	Control	CAPS
Reticulocytes (x10 ⁹ /l)	131.2	16.0***	124.9	206.4*	71.4	125.6	54.0	51.1
FMCC (x10 ⁹)	22.367	6.250***	18.923	14.412	14.790	18.038	16.815	13.014

FMCC= femoral marrow cell count; * significantly different to controls, *p<0.05; ***p<0.001

¹Robinson, S., Havard, A., Andrews, M. et al. (1999) *Hum. Exp. Toxicol.* 17, 57

²Krishna, G. et al. (1981). In: *Safety problems related to chloramphenicol and thiamphenicol*. Raven Press, New York

³Turton, J., Yallop, D., Andrews, C.M. et al. (1999) *Hum. Exp. Toxicol.* In press

Chronic hypoplastic marrow failure in the mouse; a possible new model for the assessment of drugs with potential to induce aplastic anaemia (AA)

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Aplastic anaemia (AA) is a most feared toxic effect of drugs; mortality is about 50%. Many drugs induce AA, eg chloramphenicol, phenylbutazone, phenytoin, penicillamine, gold salts. Recently, several new drugs have been withdrawn due to associations with AA, eg remoxipride (Astra), felbamate (Schering-Plough), ticlopidine (Sanofi). At present there are no pre-clinical tests for assessing the AA potential of drugs. Indeed, there are no convenient and reliable in-vivo models of drug induced AA.

However, a model of chronic hypoplastic marrow failure:AA (CHMF [AA]) was developed by Morley and Blake (1974). Busulphan (BU: 20 mg kg⁻¹) was administered to mice on 4 fortnightly occasions over 6 weeks, and the animals were studied for 240 days; although "late stage" CHMF (AA) was induced, blood counts were not greatly reduced, but marrow cell counts were decreased. Having failed to develop a reliable model of drug-induced AA (Andrews et al 1998), we have recently evaluated a new BU dosing regimen in an attempt to induce CHMF (AA) in a short period (120 days), with the objective of demonstrating "late stage" marrow depression. This was assessed by marrow colony forming assays and marrow cell counts, and significant reductions in blood erythrocytes (RBC), leucocytes (WBC) and platelets (PLT).

Female BALB/c mice were treated with BU by i.p. injection at 0 (vehicle), 5.25 and 10.50 mg kg⁻¹ on 8 occasions over 23 days. At day 1 post dosing, and at 19, 49, 91, and 112 days, mice (n = 6 to 12) in each group were sampled. A full blood count was carried out, femoral marrow flushed into IMDM medium and colony forming assays (CFU-GM and Erythroid-CFU [BFU-E + CFU-GEM]) carried out (Gibson et al 1995); humeral marrow cell counts (HMCC) were determined, marrow smears prepared, and spleens and sternums placed in fixative.

At 10.50 mg kg⁻¹, BU induced marrow depression immediately after dosing (day 1), with RBC, WBC, neutrophils, lymphocytes, monocytes, eosinophils, PLT, HMCC, CFU-GM and Erythroid-CFU significantly reduced. These parameters showed a

general trend of returning to normal at day 19 and at day 49. However, at days 91 and 112, a "late stage" marrow depression was being maintained, with significantly reduced values for WBC, lymphocytes, monocytes, eosinophils, PLT, HMCC, CFU-GM and Erythroid-CFU. At 5.25 mg kg⁻¹ BU, the pattern of effects compared with results at 10.50 mg kg⁻¹, but were less significant.

The major difference between the new BU regimen and Morley and Blake's. is that Morley administered 4 large doses of BU every 14 days over 6 weeks; with this protocol, the BU induced marrow depression would have returned towards normal at the time of the 2nd, 3rd and 4th doses (ie "infrequent zenith dosing"). In our regimen, smaller BU doses were administered, frequently, when the marrow was still depressed (ie, "frequent nadir dosing"). Here, frequent nadir dosing successfully induced late stage CHMF (AA) at days 91 and 112, with significant reductions in colony forming assays, HMCC, and blood parameters.

Morley et al (1976) later used their CHMF (AA) model to examine the haemotoxicity of chloramphenicol (CAP). After BU dosing, CAP was administered for 20 weeks: Morley reported BU-treated mice were more sensitive to CAP toxicity than non-BU-treated mice. We (Andrews et al 1998) have attempted, unsuccessfully, to induce AA with the BU+CAP protocol of Morley et al (1976). However, we consider our present frequent nadir dosing regimen an appropriate model to study the pathogenesis of AA, and the AA potential of drugs. We are preparing experiments in BU-treated mice where possible AA agents (phenylbutazone, phenytoin, penicillamine) are given from d60 post BU dosing, and animals assessed for acellular marrow and pancytopenia (AA).

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Chloramphenicol and thiamphenicol do not induce aplastic anaemia in the BALB/c mouse

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In man, chloramphenicol (CAP) induces (i), reversible anaemia developing during treatment, (ii), aplastic anaemia (AA) developing after treatment, with peripheral blood pancytopenia (PBP), and bone marrow (BM) hypocellularity (BMHC). We have developed a mouse model of CAP-induced reversible anaemia,¹ but there are no models of CAP-induced AA. We now investigate whether CAP succinate (CAPS) administered to mice at levels that cause haemotoxicity, and animals examined over an extended period, develop AA. Thiamphenicol (TAP), a related antibiotic, was included for comparison. Also, as apoptosis is increased in BM cells of patients with AA, we examined whether CAPS increases apoptosis in mouse BM mononuclear cells (BMMNC).

Weanling female BALB/c mice were gavaged daily with CAPS (2000mg/kg) or TAP (850mg/kg) for 17 days (d), at d1, 13, 22, 41, 98, 179 post dosing, blood was taken as described,² marrow smears prepared and femoral marrow cell counts completed. Haemopoietic progenitor cell cultures were prepared from femoral marrow, and colony forming assays (CFU-E, CFU-GM) carried out. Apoptotic femoral BMMNC were assayed as described,³ in 2 experiments: Expt. 1, mice were dosed (CAPS, 4000mg/kg; TAP, 4000mg/kg) and sampled at 36h; Expt. 2, mice were treated (CAPS, 1000, 2000, 3000mg/kg) and sampled at 4, 8, 16 and 32h.

Data are presented (Table). At d1, erythrocytes, Hb and haematocrits were reduced in CAPS and TAP groups. At d13 and 22, values were returning to normal. At d41, 98 and 179, treated mice generally showed normal values for all parameters, including femoral counts and M:E ratios. CFU-E and CFU-GM were reduced by CAPS and TAP at d1, 13, and 22, but were normal thereafter. Apoptosis assays in Expt. 1 showed an increase (%) in CAPS, but not TAP-treated mice (control, 19.3%; CAPS, 22.3% P<0.05; TAP, 18.6%); Expt. 2, CAPS mice showed no increase in apoptotic cells at any time, or dose, except 3000mg/kg at 4h (control, 7.7%; CAPS, 10.0% P<0.001). We conclude: CAPS and TAP induced a reversible anaemia; there was no evidence of later-developing PBP or BMHC (AA); there was no unequivocal evidence of CAPS-induced BMMNC apoptosis.

Table. Mean haematological results from BALB/c mice treated with CAPS or TAP and sampled at 1, 13, 22 and 41 days post dosing

	Day 1		Day 13		Day 22		Day 41	
	Cont	CAPS	Cont	TAP	Cont	CAPS	Cont	TAP
Hb (g/dl)	15.5	13.3 ^{***}	15.9	12.6 ^{**}	16.4	15.6 [*]	16.5	15.2
WBC ($\times 10^9/l$)	1.2	2.1	1.6	1.0	1.6	1.2	1.5	1.2
PLTS ($\times 10^9/l$)	933	1281 [*]	912	1658	895	1121 [*]	870	1273 [*]
CFU-E ¹	61.4	40.5 ^{***}	-	44.7 ^{***}	62.7	39.5 ^{***}	-	46.0 ^{***}
CFU-GM ¹	31.8	19.3 ^{***}	-	21.9 ^{***}	33.8	20.0 ^{***}	-	24.5 ^{***}
CFU-E/CFU-GM ²	1.9	2.1 ^{***}	-	2.0 ^{***}	1.8	1.8 ^{***}	-	1.9 ^{***}
M:E	1.8	1.8 ^{***}	-	1.8 ^{***}	1.8	1.8 ^{***}	-	1.8 ^{***}
Apoptosis (%) ³	7.7	19.3 ^{***}	-	18.6 ^{***}	7.7	10.0 ^{***}	-	5.6 ^{***}

Hb, haemoglobin; WBC, white blood cells; PLTS, platelets; CFU-E, colony forming units - erythroid; CFU-GM, colony forming units - granulocyte/monocytic. ¹number of colonies. n = 4 or 5 for Hb, WBC, PLTS; n = 6 or 9 cultures for CFU-E, CFU-GM. ²P<0.05; ³P<0.001.

¹Yallop, D., Andrews, M., Holt, D., York, M., Fagg, R., Williams, T. & Turton, J. (1998) *Hum. Exp. Toxicol.* 17, 57

²Holt, D., Andrews, C., Payne, J., Williams, T. & Turton, J. (1998) *Hum. Exp. Toxicol.* 17, 8-17

³Philpott, N., Turner, A., Scopes, J., Westby, M., Marsh, J., Gordon-Smith, E., Dalgleish, A. & Gibson, F. (1996) *Blood* 87, 2244-2251