Lymphocyte adhesion and activation
in inflammatory bowel diseases.
Studies relating to the effects of blockade of
alpha-4 integrins by a monoclonal antibody.

A thesis presented for the degree of Doctor in
Medicine in the University of London

Dr Fiona H Gordon
MA MBChir. MRCP(UK)

Centre for Gastroenterology
Royal Free Campus
Royal Free and University College Medical School
Rowland Hill Street
London NW3 2PF

2002
Acknowledgments

I am greatly indebted to my supervisor, Professor Roy Pounder, for his advice, wisdom and constant encouragement. I am also most grateful for the advice of other colleagues at the Royal Free Hospital including Dr Simon Murch, Dr Peter Amlot, Dr Mark Hamilton and Dr Caroline Sabin. I would also like to express thanks to Fari Tahami, Shelly Rana, Ros Sims, Kosser Khan, Professor Paul Dhillon, Judy Sercombe and Carla Blake for technical assistance.

I am grateful to Tanya Palmer, Dr Steven Donoghue and Dr Carol Greenlees at Elan Pharma Ltd. for their advice with respect to trial design and help with data collection and processing. I am also grateful to Dr Miles Allison and Marilyn Fouweather and Dr Clement Lai, Royal Gwent Hospital, Newport.

I am indebted to the Digestive Diseases Fund, the Astra Foundation and Elan Pharma Ltd., for their financial assistance with my studies.

Finally I am grateful to Robert Przemioslo, my family and friends for their unfailing support and patience.
Abstract

Alpha-4 integrins are glycoprotein mediators of activated leucocyte trafficking to the gut, which may be important in inflammatory bowel disease (IBD) pathogenesis. The studies described in this thesis aimed to assess the clinical and immunological effects of natalizumab, a humanised monoclonal antibody to α4 integrin, in patients with active IBD. A randomised double-blind placebo-controlled study of a single 3mg/kg intravenous natalizumab infusion in patients with mild to moderately active Crohn’s disease demonstrated that the drug was safe and well-tolerated. Significant reductions in disease activity scores were found post-natalizumab, but not placebo. An open study of the same dose in patients with active ulcerative colitis suggested that natalizumab may also benefit these patients. Natalizumab produced elevated circulating leucocyte counts for at least four weeks post-infusion. T cells expressing activation antigens, B cells, monocytes and eosinophils were particularly elevated, although circulating NK T cells, NK cells and gamma-delta T cells were generally unaffected, suggesting that natalizumab exerts differential effects on leucocyte trafficking in patients with active IBD. Natalizumab also produced reduced levels of the serum soluble form of its endothelial ligand, vascular cellular adhesion molecule-1 (VCAM-1), but was found to have less consistent effects on other serum soluble adhesion molecules. Consistent T cell activation antigen patterns were noted in the initial patients screened for clinical studies of natalizumab and were felt to merit further study. A wide range of lymphocyte activation markers were studied prospectively comparing patterns in newly-diagnosed and chronic IBD patients with patients with other forms of enteric inflammation and healthy volunteers. Consistent expression patterns existed between CD8+ cell activation markers and NK-type T cells in IBD patients, which were found to a lesser extent in disease control patients but not healthy volunteers. Little difference in lymphocyte activation antigen expression was found between newly-diagnosed and chronic IBD patients.
# Table of contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Table of contents</td>
<td>4</td>
</tr>
<tr>
<td>Legends to figures</td>
<td>12</td>
</tr>
<tr>
<td>Legends to tables</td>
<td>18</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>21</td>
</tr>
</tbody>
</table>

## Chapter 1  Inflammatory bowel disease

### Historical background and aetiological theories  23

1.1 Definitions  23
1.2 Crohn’s disease  23
1.3 Ulcerative colitis  23
1.4 Incidence of IBD  24
1.5 Aetiology of IBD  25
1.6 Environmental theories  25
  1.6.1 Immunological tolerance to enteric flora  25
    1.6.1.1 Evidence for the importance of enteric flora in IBD.  25
    1.6.1.2 Factors determining abnormal tolerance to enteric flora  26
  1.6.2 Other possible environmental triggers of IBD  27
    1.6.2.1 Microbial factors  27
    1.6.2.2 Non-microbial dietary triggers  28
    1.6.2.3 Smoking  28
    1.6.2.4 Miscellaneous environmental triggers  28
1.7 Genetics and IBD  29
  1.7.1 Family studies  29
  1.7.2 Gene mapping by linkage analysis  29
  1.7.3 Genetic polymorphisms of candidate genes  30
1.8 Vasculopathic hypotheses and IBD  31
  1.8.1 Vasculitis and ischaemia  31
  1.8.2 Measles virus  31
Chapter 2  Immunological factors and IBD pathogenesis...

2.1 Evidence that T cells are important effector cells in IBD

2.1.1 Evidence from studies of mucosal T cells

2.1.2 Evidence from studies of peripheral blood

2.1.3 Evidence from studies of T cell receptor subtypes

2.1.4 Evidence of T cell importance from animal models of IBD

2.1.5 Evidence for T cell importance from clinical studies

2.2 The relationship between T cells and other mediators

2.2.1 Cytokines: an overview

2.2.2 Pro-inflammatory cytokines

2.2.3 The role of TNFα in IBD; evidence from anti-TNFα therapies

2.2.3.1 Clinical trials of infliximab

2.2.3.2 Clinical trials of CDP571

2.2.3.3 Etanercept

2.2.3.4 Other anti-TNFα therapies in IBD

2.2.4 Evidence from clinical trials of the importance of ‘anti-inflammatory’ cytokines in IBD

2.2.4.1 Interleukin 10

2.2.4.2 Interleukin 11

2.2.5 Nuclear transcription factors

2.2.6 Extra-cellular matrix components

2.2.7 Eicosanoids

2.2.8 Reactive oxygen and nitrogen metabolites

2.2.9 Growth factors

2.3 Leucocyte adhesion and IBD

2.3.1 Introduction

2.3.2 Selectins and leucoocyte rolling
2.3.3 Integrins, leucocyte adherence and emigration ................. 47
2.3.4 Beta-2 integrins ............................................................... 47
2.3.5 Animal studies of alpha-4 integrins ................................... 48
2.3.6 Clinical studies of alpha-4 integrins .................................... 49
2.3.7 Alpha-E integrins, cadherins and catenins ......................... 50
2.3.8 Control of adhesion molecule expression: the role of TNF\(\alpha\) and NFkB ................................................................. 50
2.3.9 Effects of IBD therapies on adhesion molecule expression ... 51
2.3.10 Summary of leucocyte adhesion mechanisms ................. 51
2.4 Natalizumab ........................................................................ 53
   2.4.1 Background ................................................................. 53
   2.4.2 Studies of natalizumab in mammals ......................... 53
   2.4.3 Clinical studies of natalizumab ................................... 54

Chapter 3 Clinical trial methods in IBD ............................... 55
3.1 Patient selection ................................................................... 55
   3.1.1 Disease definition ......................................................... 55
   3.1.2 Disease extent .............................................................. 56
   3.1.3 Disease activity for trial entry ....................................... 56
       3.1.3.1 Inducing remission of active disease ...................... 57
       3.1.3.2 Maintenance treatment trials .................................. 57
   3.1.4 Exclusion criteria ........................................................ 58
       3.1.4.1 Patient-related issues .............................................. 58
       3.1.4.2 Drug-related issues ............................................... 59
3.2 Study design ......................................................................... 60
   3.2.1 Sample size ................................................................. 60
   3.2.2 Randomisation and choice of comparisons .................... 60
   3.2.3 Stratifying groups ........................................................ 61
   3.2.4 Blinding ....................................................................... 61
   3.2.5 Trial end-points ............................................................ 62
3.3 Data collection ...................................................................... 63
   3.3.1 Crohn’s disease activity scores .................................... 64
   3.3.2 Ulcerative colitis activity scores .................................. 65
   3.3.3 Quality of life measures in IBD ................................. 65
3.3.4 Endoscopic score systems....................................................... 66
3.3.5 Evaluation of histology in IBD trials..................................... 67
3.3.6 Safety data and adverse events.............................................. 67
  3.3.6.1 Treatment-specific events........................................... 67
  3.3.6.2 Disease-related events................................................. 68
3.4 Concomitant medication.......................................................... 69
  3.4.1 Pre-trial medication............................................................ 69
  3.4.2 Management of existing medication................................. 69
  3.4.3 Rectal treatments............................................................... 70
  3.4.4 Rescue medication............................................................. 70
3.5 Data-handling............................................................................. 70
  3.5.1 Missing data....................................................................... 71
  3.5.2 Missing CDAI data............................................................. 71

Chapter 4  A randomised placebo-controlled trial of natalizumab,
a humanised monoclonal antibody to alpha-4 integrin
in active Crohn’s disease......................................................... 73
4.1 Abstract .................................................................................... 73
4.2 Introduction............................................................................... 74
4.3 Methods.................................................................................... 74
  4.3.1 Patients............................................................................... 74
  4.3.2 Exclusion criteria................................................................. 75
  4.3.3 Design................................................................................. 75
  4.3.4 Statistical analysis............................................................... 76
4.4 Results....................................................................................... 76
  4.4.1 Demography........................................................................ 76
  4.4.2 Clinical response to treatment........................................... 79
  4.4.3 Quality of life..................................................................... 81
  4.4.4 Inflammatory markers......................................................... 81
  4.4.5 Pharmacokinetics and antibody formation........................ 82
  4.4.6 Tolerability and adverse events.......................................... 82
  4.4.7 Immunological parameters................................................ 83
4.5 Discussion.................................................................................. 85
8.4.3 Concurrent treatment ................................................................. 129
8.4.4 Basic lymphocyte subsets .......................................................... 130
8.4.5 Lymphocyte activation marker expression ............................... 130
8.4.6 Patterns of activation marker expression .................................... 133
8.4.6.1 CD45RO+ cells ................................................................. 133
8.4.6.2 CD8CD28+ cells .............................................................. 134
8.4.6.3 CD8DR+ T cells ............................................................... 135
8.4.6.4 NK cells (CD16+) and NK T cells (CD3+CD57+). ..................... 136
8.4.7 Unusual findings in individual patients ..................................... 138
8.5 Conclusions ................................................................................... 138

Chapter 9 Discussion ................................................................. 140

9.1 Natalizumab clinical studies .......................................................... 140
9.1.1 Safety and tolerability .............................................................. 140
9.1.2 Immunogenicity ....................................................................... 140
9.1.3 Infection risk ........................................................................... 141
9.2 Efficacy of natalizumab in IBD ...................................................... 141
9.2.1 The impact of pharmacokinetics on efficacy ............................. 141
9.2.2 Patient characteristics and response ....................................... 143
9.2.3 Inflammatory markers and response ....................................... 144
9.3 Trial design learning points from Crohn’s disease studies .......... 144
9.3.1 End-points .............................................................................. 144
9.3.2 CDAI limitations .................................................................... 145
9.4 Learning points in trial design from ulcerative colitis study ....... 145
9.4.1 Use of scoring systems in ulcerative colitis trials ................... 145
9.4.2 Use of rectal medication .......................................................... 146
9.5 Immunological effects of natalizumab .......................................... 147
9.5.1 Effects on basic leucocyte sub-types ........................................ 147
9.5.1.2 Lymphocytes ................................................................ 147
9.5.1.2 Other leucocyte subsets ................................................... 147
9.5.1.3 Comparison with other studies ........................................ 148
9.5.2 Effects on adhesion molecule expression ................................. 148
9.5.2.1 α4 integrin and VCAM-1 interactions ................................. 148
9.5.2.2 ICAM-1 interactions ......................................................... 150
9.5.2.3 Selectin pathways........................................................ 150
9.5.3 Effects of natalizumab on other lymphocyte subsets and
NK cells.................................................................................... 150
  9.5.3.1 NK cells........................................................................ 150
  9.5.3.2 Gamma-delta T cells.................................................... 151
  9.5.3.3 Activated T cells.......................................................... 151

9.6 FACS analysis of peripheral blood lymphocytes of newly-diagnosed
versus chronic IBD patients.......................................................... 152
  9.6.1 Comparison between IBD patients and controls.............. 152
  9.6.2 Comparison between patients with newly-diagnosed and
chronic IBD............................................................................... 153
  9.6.3 Correlation between different lymphocyte activation markers 154
  9.6.4 Individual patient anomalies............................................ 154

Chapter 10 Conclusions and plans for future work.................156
10.1 Conclusions.................................................................................. 156
10.2 Future studies................................................................................ 157
  10.2.1 Clinical studies of natalizumab in IBD patients.............. 157
  10.2.2 Immunological effects of natalizumab............................ 158
  10.2.3 Patterns of activation antigen expression in IBD patients.... 159

Appendix I Sample Crohn's disease activity index (CDAI) score sheet. 161
Appendix II Sample Powell-Tuck activity index score sheet.......... 162
Appendix III Monoclonal antibodies used for FACS analysis and suppliers of
other reagents............................................................................ 163
Appendix IV Precision of serum adhesion molecule ELISAs........... 166
Appendix V Copies of Ethics Committee correspondence for Chapters
  4, 5 and 8.................................................................................. 167
Bibliography..................................................................................... 174
Publications related to this work..................................................... 211
Practical contributions to this thesis from other workers................. 213
Legends to figures

Figure 1 52 Schematic diagram to illustrate the effect of natalizumab on α4 integrin-mediated leucocyte adhesion.

4.1 79 Trial profile of patients with mild to moderately active Crohn’s disease who received 3mg/kg natalizumab or placebo.

4.2 80 Mean CDAI by patient group post-infusion. Mean CDAI of Crohn’s disease patients post-infusion of 3mg/kg natalizumab or placebo. Bars indicate SD and ** denotes significant changes compared to baseline (p<0.05). Numbers above and below bars indicates the number of rescued patients at each time-point.

4.3 81 Clinical status of Crohn’s disease patients two weeks after single infusion of natalizumab 3mg/kg or placebo. Observed differences between groups are not significant.

4.4 82 Mean serum natalizumab concentrations of 18 Crohn’s disease patients following a single 3mg/kg infusion. Bars indicate SD.

4.5 84 Mean lymphocyte counts post-natalizumab or placebo. ** p<0.01 compared to baseline values. Bars are SD.

4.6 84 Changes in B and T cells in natalizumab-treated patients. ** p<0.0001 and * p<0.01 compared to baseline.

5.1 93 The effects of natalizumab on Powell-Tuck score in 10 patients with active ulcerative colitis. Powell-Tuck activity scores of 10 ulcerative colitis patients after single 3mg/kg natalizumab infusion. Boxes around data-points indicate time at which rescue medication was commenced.
Figure 5.2 95 CRP and ESR values post-natalizumab in 10 ulcerative colitis patients. Median CRP and ESR values post 3mg/kg natalizumab. Bars are SD.

5.3 95 Mean serum concentrations of natalizumab in 10 patients with active ulcerative colitis. Serum natalizumab concentrations after 3mg/kg infusion. Bars are SD.

5.4 96 Lymphocyte counts post-natalizumab in 10 ulcerative colitis patients. Mean lymphocyte counts after 3mg/kg natalizumab infusion. Bars are SD and ** (p<0.005) and * (p<0.05) indicate significant changes compared to baseline.

5.5 97 Quality of life in ulcerative colitis patients after natalizumab. IBDQ quality of life scores of eight ulcerative colitis patients (with complete data-sets) before and after 3mg/kg natalizumab infusion.

6.1a 104 The effect of natalizumab on circulating eosinophils in patients with active Crohn’s disease and ulcerative colitis. Significantly increased circulating eosinophil counts in Crohn’s disease (n=18) and ulcerative colitis patients (n=10) after 3mg/kg natalizumab infusion.

6.1b 104 The effect of natalizumab on circulating monocytes in patients with active Crohn’s disease and ulcerative colitis. Significantly increased monocyte counts in active Crohn’s disease and ulcerative colitis patients after 3mg/kg natalizumab infusion.
Figure Page

6.2a 105 The effects of natalizumab on TCRαβ+ cells expressing activation antigens in patients with active Crohn’s disease. Significantly increased TCRαβ+ cells expressing CD26, HLA-DR, CD8DR and CD8CD28 to at least four weeks post 3mg/kg natalizumab infusion.

6.2b 105 The effects of natalizumab on TCRαβ+ cells expressing activation antigens in patients with active ulcerative colitis. Significantly increased TCRαβ+ cells expressing CD26, HLA-DR, CD8DR and CD8CD28 to at least four weeks post 3mg/kg natalizumab infusion.

6.2c 106 The effects of natalizumab on TCRαβ+ cells expressing activation antigens and memory and naïve markers in patients with active Crohn’s disease. Significantly increased memory (CD45RO+) and naïve (CD45RA+) TCRαβ+ cells to at least four weeks after 3mg/kg natalizumab infusion in Crohn’s disease patients.

6.2d 106 The effects of natalizumab on TCRαβ+ cells expressing activation antigens, memory and naïve markers in patients with active ulcerative colitis. Significantly increased memory (CD45RO+), naïve (CD45RA+), CD69+ and CD38+ TCRαβ+ cells at one week after 3mg/kg natalizumab infusion in ulcerative colitis patients.

6.3a 107 The effects of natalizumab on circulating TCRγδ+ and NK-type cells in patients with active Crohn’s disease.

6.3b 107 The effects of natalizumab on circulating TCRγδ+ and NK-type cells in patients with active ulcerative colitis. Overall lack of significant effect of 3mg/kg natalizumab infusion on TCRγδ+ and NK-tvoe cells in patients with active IBD.
The effect of natalizumab on monoclonal kappa light chain+ TCRαβ+ cells in patients with active Crohn’s disease (n=18) and ulcerative colitis (n=10). Significant elevation in TCRαβ+ kappa light chain+ cells after natalizumab.

Comparison of serum soluble adhesion molecule concentrations between disease groups at baseline. Graph showing no significant difference between controls, patients with active Crohn’s disease and patients with active ulcerative colitis at baseline. Columns show mean serum adhesion molecule concentrations +/- SD.

The effect of natalizumab on serum VCAM-1 concentrations in patients with active Crohn’s disease (n=8) and ulcerative colitis (n=10). Lines are means +/- SD. *=significance compared to baseline.

The effect of natalizumab on serum ICAM-1 concentrations in patients with active Crohn’s disease and ulcerative colitis. No significant changes in serum ICAM-1 concentrations at one and two weeks after 3mg/kg natalizumab infusion in Crohn’s disease (n=8) and ulcerative colitis (n=10) patients.

The effect of natalizumab on serum E-selectin concentrations in patients with active Crohn’s disease and ulcerative colitis. No significant changes in E-selectin levels in Crohn’s disease or ulcerative colitis patients.

Serum soluble adhesion molecule concentrations in Crohn’s disease patients who received placebo. No significant differences found in serum VCAM-1, ICAM-1 or E-selectin concentrations in Crohn’s disease patients who received placebo.
Figure 7.6 120  
Comparison of circulating α4+ TCRαβ+ lymphocyte counts between IBD patients and controls at baseline. Patients with active Crohn's disease (n=15) had significantly lower baseline CD49d+ cell counts than controls (n=8).

Figure 7.7 120  
The effects of natalizumab on circulating TCRαβ+ lymphocytes which express α4 integrins (CD49d+) in patients with active IBD. Significant increase in circulating CD49d+ (α4 integrin+) cells compared to baseline at one week after 3mg/kg natalizumab infusion in ulcerative colitis patients only. Values are means +/- SD.

Figure 7.8 121  
The effects of natalizumab on circulating TCRαβ+ cells which express ICAM-1 (CD54+) in patients with active IBD. Significantly increased peripheral CD54+ TCRαβ+ cells in patients with active IBD at one and two weeks after 3mg natalizumab infusion.

Figure 7.9 121  
The effects of natalizumab on circulating TCRαβ+ lymphocytes which express L-selectin (CD62L+) in patients with active IBD. Significantly increased peripheral CD62L+ T cells in ulcerative colitis (n=10) and Crohn's disease patients (n=8) at one and two weeks after a single 3mg/kg natalizumab infusion, respectively. * = p<0.05 compared to baseline.

Figure 7.10 122  
The effects of placebo on circulating TCRαβ+ cells expressing CD49d (α4 integrin), CD54 (ICAM-1) and CD62L (L-selectin) in patients with active Crohn's disease. Significant reduction in CD54+TCRαβ+ cells at one week post-placebo only. No significant changes in other T cell subsets.
8.1 132 Comparison of lymphocyte subsets in patients with newly-diagnosed and chronic Crohn’s disease. Comparison of T cell (TCRαβ+), B cell (CD19+) and NK cell (CD16+) proportions in patients with Crohn’s disease. Significant differences are shown between new and chronic patients. Error bars are SD.

8.2 132 Comparison of lymphocyte subsets in patients with newly-diagnosed and chronic ulcerative colitis. Comparison of T cell (TCRαβ+), B cell (CD19+) and NK cell (CD16+) proportions in patients with ulcerative colitis. Significant differences are shown between new and chronic patients. Error bars are SD.
Legends to tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>78</td>
<td>Demographic characteristics of Crohn’s disease patients at study-entry</td>
</tr>
<tr>
<td>5.1</td>
<td>92</td>
<td>Demographic characteristics of ulcerative colitis patients at study-entry</td>
</tr>
<tr>
<td>5.2</td>
<td>96</td>
<td>Spearman correlations between change in Powell-Tuck score and change in total lymphocyte counts in 10 ulcerative colitis patients. *Recorded data used for these analyses.</td>
</tr>
<tr>
<td>5.3</td>
<td>97</td>
<td>Sigmoidoscopic scores of 10 patients who received natalizumab. 0=normal, 1=granular mucosa, 2=contact/spontaneous bleeding, 3=severe changes +/- ulceration. Red text indicates rescue therapy commenced.</td>
</tr>
<tr>
<td>6a</td>
<td>109</td>
<td>Leucocyte subsets in placebo-treated Crohn’s disease patients. Mean (SD) leucocyte subset values post-placebo; no significant differences in any group compared to baseline (week 0) values.</td>
</tr>
<tr>
<td>6b</td>
<td>109</td>
<td>T cell and NK markers in placebo-treated Crohn’s disease patients. Columns show mean counts (SD) of T cell subsets and NK-type cells of Crohn’s disease study placebo patients compared with baseline values (Wilcoxon signed rank test; p&lt;0.05). Significant differences compared to baseline are shown in bold. Patients who received rescue corticosteroids were not included in the analysis from the point at which treatment commenced.</td>
</tr>
</tbody>
</table>
Spearman r values comparing lymphocyte subsets and disease activity. No significant correlation found between lymphocyte subset counts and CDAI (Crohn’s disease patients) or Powell-Tuck score (ulcerative colitis patients).

Spearman r values comparing lymphocyte subsets with serum natalizumab. No significant correlation between disease activity and lymphocyte subsets, except for total lymphocyte counts at week 4 (p=0.04).

Patient demography

Treatment by group

Basic lymphocyte subsets. Numbers are means +/-SD. Subsets are expressed as percentage of total lymphocyte counts.

Lymphocyte activation antigen expression. Numbers marked* are expressed as percentages of TCRαβ+ lymphocyte counts with SD in italics. Numbers marked ** are percentage of total CD8+ cells and remainder are percentage of total lymphocyte counts.

Correlation between CD45RO+ T cells and other subsets.

R = correlation coefficient for values where p<0.05.

Correlation between CD8+CD28+ T cells and other subsets. R = correlation coefficient for values where p<0.05.

Correlation between CD8+DR+ T cells and other subsets.

R = correlation coefficient for values where p<0.05.
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
</table>
| 8.5d  | 137  | Correlation between CD57+ T cells and other subsets.  
<pre><code>  |      | R = correlation coefficient for values where p&lt;0.05. |
</code></pre>
<p>| 8.5e  | 137  | Correlation between NK cells (CD16+) and other subsets. |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANCA</td>
<td>anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASA</td>
<td>amino-salicylic acid</td>
</tr>
<tr>
<td>CDAI</td>
<td>Crohn’s disease activity index</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell-sorter analysis</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>HACA</td>
<td>human anti-chimeric antibody</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>IBDQ</td>
<td>inflammatory bowel diseases questionnaire</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LOCF</td>
<td>last observation carried forward</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAAdCAM</td>
<td>mucosal addressin cellular adhesion molecule</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
</tr>
<tr>
<td>RFH</td>
<td>Royal Free Hospital</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROM</td>
<td>reactive oxygen metabolite</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzene sulphonic acid</td>
</tr>
<tr>
<td>VLA</td>
<td>very late antigen</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>tissue inhibitor of metalloprotease-1</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
</tbody>
</table>
Chapter 1
Inflammatory bowel disease: historical background
and aetiological theories

1.1 Definitions
Inflammatory bowel diseases are chronic inflammatory conditions of the human
gut of unknown aetiology. The commonest inflammatory bowel diseases are
Crohn’s disease and ulcerative colitis, but other rarer conditions include
lymphocytic colitis, microscopic colitis, collagenous colitis, eosinophilic colitis
and colitides associated with multi-system disorders, such as Behcet’s disease and
congenital immunodeficiency syndromes. Throughout this work, the term
inflammatory bowel disease (IBD) will be used to refer to Crohn’s disease and
ulcerative colitis.

1.2 Crohn’s disease
Crohn’s disease is characterised clinically by the presence of patchy, granulomatous transmural ulceration of the gastro-intestinal tract (GI-tract).
Several disease phenotypes are described which relate to the pattern of disease
distribution in the GI-tract, none of which are mutually exclusive. Most patients
(80%) experience disease involving the terminal ileum which may extend to the
caecum and ascending colon, and many patients have patchy disease of the colon.
Disease limited to the small bowel occurs in approximately 30% of patients
compared with isolated colonic disease in 25% of patients, whilst 30% of patients
have peri-anal disease (Farmer, Hawk, et al. 1975). Gastric, duodenal and
oesophageal involvement are well-recognised, although rarely account for
patients’ main symptoms.

1.3 Ulcerative colitis
In contrast to Crohn’s disease, ulcerative colitis results in chronic inflammation
superficial to the muscularis mucosae of the colon and rectum only. Mucosal
lesions are characteristically continuous, extending a variable distance from the
rectum proximally to the ileo-caecal valve, with terminal ileal ‘back-wash’ ileitis
occurring rarely. The commonest pattern of disease at presentation is left-sided
disease (50%) affecting the descending colon distal to the splenic flexure, with involvement of the entire colon (total or pancolitis) or isolated rectal inflammation (proctitis) each occurring in 25% of patients.

1.4 Incidence of IBD
Crohn’s disease was first characterised by Burril Crohn and others in 1932 {Crohn, Ginzburg, et al. 1932}, although Dalziel had reported a case of ‘regional ileitis’ in Glasgow in 1913, which also may have been Crohn’s disease. Allchin reported the earliest series of patients with ulcerative colitis {Allchin W.H. 1909}, having published the first case report of the disease some years earlier {Allchin 1885}. Its incidence has reached a constant rate since first reported {Calkins, Lilienfeld, et al. 1984} and in some areas it appears to have reduced with increasing westernisation {Kyle 1992}.

The incidence of Crohn’s disease has increased steadily over the twentieth century, particularly in northern European countries and North America {Gollop, Phillips, et al. 1988} {Hildebrand, Brydolf, et al. 1994} {Montgomery, Morris, et al. 1998}, provoking speculation that changing environmental factors may be important in determining its incidence. There is wide geographic variation of IBD incidence from less than 1/100,000 and 2/100,000 inhabitants per annum in South American nations {Linares de la Cal JA, Canton, et al. 1999} to 12/100,000 and 7/100,000 inhabitants per annum in westernised countries such as Sweden and the UK, for Crohn’s disease and ulcerative colitis, respectively {Ekborn, Helmick, et al. 1991} {Kyle 1992}. Migrating populations tend to adopt the incidence of IBD of their destination, for example Cantonese immigrants to Vancouver have a higher prevalence of Crohn’s disease than those resident in Hong Kong itself {Chaun, Kwan, et al. 1998}. A similar effect has been reported amongst Asian immigrants to the UK {Feehally, Burden, et al. 1993}.

In addition to this documented rise in incidence of IBD, the age of onset appears to be decreasing from the third to the first or second decade of life. In Scotland for example, the incidence of juvenile-onset Crohn’s disease doubled between 1982 and 1992 {Armitage, Drummond, et al. 1999}. The prevalence of IBD has a slight female predominance, with a female to male ratio of approximately two to one,
whereas the reverse is true for ulcerative colitis. There is no clear explanation for this observation, although it is in keeping with the general female predilection for chronic immune-mediated diseases such as rheumatoid arthritis and auto-immune conditions.

1.5 Aetiology of IBD
The plethora of hypotheses for the aetiology of IBD supports the argument that its cause is likely to be multifactorial. The importance of genetic factors is suggested by the familial nature of the disease and the increased likelihood of monozygotic twins developing IBD compared to those who are dizygotic {Thompson, Driscoll, et al. 1996}. However, the changes in incidence outlined above cannot be explained by genetic factors alone and it is more likely that IBD represents an abnormal, possibly genetically determined, response to environmental factors. Additionally, both the disease incidence and the frequency of exacerbations have been noted to display seasonal variation, supporting the importance of environmental factors {Ekbom, Zack, et al. 1991}{Sonnenberg, Jacobsen, et al. 1994}.

1.6 Environmental theories

1.6.1 Immunological tolerance to enteric flora
1.6.1.1 Evidence for the importance of enteric flora in IBD
Animal studies have provided some of the most convincing evidence that host flora may have an aetiological role in IBD. Transgenic mice deficient either in interleukin-2 (IL-2) or interleukin-10 (IL-10) have been shown to develop colitis not dissimilar from IBD, but do not develop disease if raised in a germ-free environment {Sadlack, Merz, et al. 1993}{Kuhn, Lohler, et al. 1993}. Studies of mice with severe combined immunodeficiency (SCID) replete with CD45RB\textsuperscript{high}+CD4+ lymphocytes have demonstrated similar results, in that infection with \textit{Helicobacter hepaticus} is sufficient alone to induce IBD {Cahill, Foltz, et al. 1997}.

IBD patients have been found to have a heightened mucosal IgG response to enteric flora which returns to normal control levels once the disease is in remission {Macpherson, Khoo. et al. 1996}. Studies of antibody responses to
Saccharomyces cervisiae {Main, McKenzie, et al. 1988}, Klebsiella {Tiwana, Walmsley, et al. 1998}, and E. Coli {Duchmann, Kaiser, et al. 1995} have also demonstrated that IBD patients have heightened immune responses to these micro-organisms, suggesting that abnormal immune tolerance is important in IBD pathogenesis. However, the presence of an increased humoral response to enteric flora does not prove causality and may represent a broadly enhanced immune response.

Other workers have suggested that IBD patients have altered gut flora constituents, rather than a heightened response to standard commensal organisms. For instance, studies comparing the enteric flora of patients with Crohn’s disease, healthy controls and patients who have had curative large intestinal resection for colorectal cancer, demonstrate that Crohn’s patients’ flora has a significantly lower proportion of constituent anaerobic Bifidobacterium and a higher proportion of aerobic Enterobacteria species than controls {Neut, Colombel, et al. 1989}{Favier, Neut, et al. 1997}. Reduced frequency of disease relapses in Crohn’s and ulcerative colitis patients treated with oral ‘probiotic’ bacteria compared to placebo also suggest that altering the complement of gut flora may be beneficial {Rembacken, Snelling, et al. 1999}{Guslandi, Mezzi, et al. 2000}. Together, these findings support the hypothesis that a state of abnormal tolerance to host bacteria exists in IBD. Whether this abnormal process is causal, or a secondary consequence of a chronically breached enteric mucosa remains unclear. In addition, the factors responsible for this abnormal tolerance remain to be elucidated.

1.6.1.2 Factors determining abnormal tolerance to enteric flora
Immunological tolerance to environmental antigens is critical for the development of normal mucosal immunity. The GI and respiratory tract mucosae of infants and young children form the prime routes of initial exposure to a wide range of antigenic stimuli. With respect to the gut, it has been suggested that a sufficiently high antigenic load or dose is required to eliminate the development of an abnormal host response to enteric flora {Friedman & Weiner 1994}. A variety of hypotheses exist as to why normal tolerance might fail to develop in IBD. Firstly, it has been suggested that progressively increased hygiene standards in infancy
may be a potential cause of suboptimal antigenic challenge to the gut mucosa. This is supported by the observation that IBD incidence is increased in westernised countries and is inversely proportional to infant mortality rates in Europe {Montgomery, Pounder, et al. 1997}.

Increased use of antibiotics for infections in early childhood has also been suggested as a potential cause of altering exposure to environmental antigens and has been found to be positively associated with subsequent development of IBD {Wurzelmann, Lyles, et al. 1994}. Childhood vaccination has been proposed as an iatrogenic means of altering gut mucosal tolerance. For example, measles vaccine has been shown to affect cell-mediated immunity {Lisse, Aaby, et al. 1994} and has been proposed to facilitate persistence of enteric measles infection, resulting in IBD {Thompson, Montgomery, et al. 1995}; {Wakefield, Sim, et al. 1997}. Appendicectomy has been shown to delay the development of disease in a mouse model of colitis {Bhan, Mizoguchi, et al. 2000} and such an association has also been reported as being protective against ulcerative colitis in IBD case-control studies {Duggan, Usmani, et al. 1998}. A hypothesis for the mechanism by which appendicectomy might protect against colitis, is that it prevents the development of abnormal tolerance by removing one of the most immunologically active areas of the gut, namely Peyer’s patch mucosa.

1.6.2 Other possible environmental triggers of IBD

1.6.2.1 Microbial factors

*Mycobacterium paratuberculosis* is known to cause a granulomatous disease in cattle which bears some similar features to IBD, and has been proposed as a putative cause of Crohn’s disease by Hermon-Taylor et al. His group found evidence of increased mycobacterial genomic DNA in colonic tissue of patients with IBD compared to healthy controls {Sanderson, Moss, et al. 1992}. Furthermore, the same group suggested that anti-tuberculous treatment could be of benefit in maintaining remission of Crohn’s disease {Hampson, Parker, et al. 1989}. Other workers, however, have been unable to repeat the results of both the tissue analyses {Rowbotham, Mapstone, et al. 1995} and the clinical studies of anti-tuberculous therapy {Borgaonkar, MacIntosh, et al. 2000}, thus the hypothesis remains controversial. With respect to ulcerative colitis, the presence
of abnormally high levels of enteric sulphur-producing bacteria species has been suggested as promoting colonic inflammation {Pitcher & Cummings 1996}.  

1.6.2.2 Non-microbial dietary triggers
Enhanced exposure to modern dietary additives, such as titanium dioxide and alumino-silicates, has been suggested as being an important in IBD aetiology {Powell, Harvey, et al. 2000}. Positive results of exclusion diets in Crohn’s patients also suggest that the antigenic load of food itself may perpetuate inflammation, although no individual foodstuff has been identified as being particularly responsible {Riordan, Hunter, et al. 1993}. Other proposed dietary triggers include fluoride, dairy products and cornflakes, none of which have been substantiated by epidemiological studies.

1.6.2.3 Smoking
The paradoxical relationship between smoking tobacco and IBD is well-recognised, in that smoking is associated with increased disease activity and early post-operative relapse in Crohn’s disease patients, but by contrast, smoking cessation is associated with the onset of ulcerative colitis {Calkins 1989}. The precise components responsible for these contrasting effects are unknown, but may be related to nicotine. In vitro, nicotine has been shown to inhibit leucocyte adhesion to endothelial cell lines {Bhatti, Haskard, et al. 1997} and to stimulate mucin production by colonic mucosa {Finnie, Campbell, et al. 1996}. In addition, nicotine has been shown to reduce levels of some colonic proinflammatory cytokines (IL-1β and IL-8) in ulcerative colitis patients without affecting intestinal mucosal permeability {Benoni & Prytz 1998}. Finally, nicotine has been found to reduce colonic smooth muscle tone, although how this might relate to inflammatory bowel disease is not clear {Green, McKirdy, et al. 1999}. In vivo, nicotine has been shown to be of therapeutic benefit in ulcerative colitis, when administered as skin patches or enemas {Pullan, Rhodes, et al. 1994}{Sandborn, Tremaine, et al. 1997}.

1.6.2.4 Miscellaneous environmental triggers
Studies of twins discordant for IBD have been used to assess possible environmental differences in early life which might account for the difference in
disease expression. The results of these studies suggest that increased contact with animals, atopy and increased frequency of gastroenteritis as a child (i.e. in advance of IBD development) are positively associated with IBD {Subhani, Montgomery, et al. 1997}.

1.7 Genetics and IBD

1.7.1 Family studies
The familial nature of IBD is well-recognised, as is its increased prevalence in certain ethnic groups, such as Jewish people. The life-time risk of a first degree relative of an affected proband developing Crohn’s disease or ulcerative colitis respectively, is predicted as 5.2% and 1.6% for non-Jewish people, compared with 7.8% and 4.5% for Jewish people {Yang, McElree, et al. 1993}. Twin studies have demonstrated that concordance rates are greater for Crohn’s disease than for ulcerative colitis, the rates being higher for monozygotic than dizygotic twins, or twins of unknown zygosity {Tysk, Lindberg, et al. 1988}. In addition, Crohn’s disease has been observed to present at the same age and be of similar phenotype in monozygotic twins {Rampton & Stott 1986}. By contrast, the relative risk of a dizygotic unaffected twin developing IBD is similar to that for an unaffected sibling {Subhani, Montgomery, et al. 1998}. Lastly, it has been proposed that children of an affected parent develop IBD at an earlier age, a phenomenon termed ‘genetic anticipation’{Satsangi, Grootscholten, et al. 1996}.

1.7.2 Gene mapping by linkage analysis
Many workers have been prompted to seek candidate genes involved in IBD pathogenesis, following rapid advances in molecular genetics, the discovery of genetic ‘knockout’ animal models of IBD and recognition of the familial nature of IBD. Early studies involved analyses of large series of families, in whom at least two first degree relatives were affected by IBD. Initial linkage analyses suggested that chromosomes 3, 7, 12 and 16 contained potential candidate genes for IBD {Satsangi, Parkes, et al. 1996} {Hugot, Laurent-Puig, et al. 1996}. A subsequent study of 274 European kindreds reproduced the association between chromosomes 12 and 16 with IBD {Curran, Lau, et al. 1998} and one further study suggested an association between chromosome 16 and ulcerative colitis, specifically {Mirza, Lee, et al. 1998}. Further studies of chromosome 16 in IBD patients have yielded
perhaps one of the greatest breakthroughs in IBD genetics to date. Two independent groups have identified a frameshift variant and missense mutations of the NOD2 gene on chromosome 16, which appears to confer susceptibility to Crohn’s disease {Hugot, Chamaillard, et al. 2001}{Ogura, Bonen, et al. 2001}. The wild-type NOD2 gene product activates NF-κB and is important in recognising bacterial lipopolysaccharide. It is not yet clear how interrupting this mechanism leads to increased susceptibility to Crohn’s disease.

Whether IBD is linked to abnormalities of the gene for the major histocompatibility complex or HLA haplotype on chromosome 6 remains controversial {Naom, Lee, et al. 1996}{Satsangi, Welsh, et al. 1996}. More recent studies have suggested that IBD may be associated with abnormalities of the X chromosome and chromosome 6p {Hampe, Schreiber, et al. 1999}{Hampe, Shaw, et al. 1999}. Linkage analyses of genetically homogenous populations with a high incidence of IBD, such as Iceland, are likely to further understanding of potential candidate genes.

1.7.3 Genetic polymorphism of candidate genes

The alternative analytic approach to a genome-wide search for susceptibility genes is to select likely candidate genes for analysis of polymorphisms, using the hypothesis that IBD may be due to abnormal expression of a regulatory protein. Virtually all of such studies have focussed on genetic polymorphisms of cytokines and have yielded conflicting results. An example of this controversy are early studies of genetic polymorphisms of the interleukin-1 receptor antagonist (IL-1ra) which showed no association with IBD {Louis, Satsangi, et al. 1996}{Hacker, Gomolka, et al. 1997}. More recent studies suggest that some polymorphisms of this gene may indeed be associated with IBD, but that the association varies with ethnicity {Tountas, Casini-Raggi, et al. 1999}.

Tumour necrosis factor alpha (TNFα) gene polymorphisms have also been studied extensively. A recent study of European kindreds has found the TNFα2 allele to be associated with steroid-resistant IBD and fistulising Crohn’s disease {Louis, Peeters, et al. 2000}. Previous studies concur with these findings and furthermore, suggest that this specific TNFα polymorphism is positively associated with the
presence of serum anti-neutrophil cytoplasmic antibodies (ANCA) \{Satsangi, Landers, et al. 1998\} \{Hirv, Seyfarth, et al. 1999\}. To date, studies of polymorphisms of genes for interleukins 4 and 10 have been largely negative \{Parkes, Satsangi, et al. 1998\} \{Olavesen, Hampe, et al. 2000\}.

Finally, the MUC3 polymorphism of the mucin gene on chromosome 7 has been reported to be associated with ulcerative colitis \{Kyo, Parkes, et al. 1999\} and expression of MUC3 messenger RNA (mRNA) has been found to be reduced in Crohn’s disease, suggesting that dysregulation of mucin genes may be important in IBD development \{Buisine, Desreumaux, et al. 1999\}.

### 1.8 Vasculopathic hypotheses and IBD

#### 1.8.1 Vasculitis and ischaemia

Studies of resin casts of the mesenteric vasculature of intestinal resection specimens from Crohn’s disease patients led to the hypothesis that IBD is primarily a vasculitis, since areas of mucosal ulceration and granulomata correlated strongly with the presence of infarction at the muscularis propria level \{Wakefield, Sawyerr, et al. 1989\}. Similar analyses of ulcerative colitis patients suggested that the proximal extent of the disease is limited by the distribution of the marginal artery \{Hamilton, Dick, et al. 1995\}. Finally, detailed anatomical studies of resected small bowel specimens from Crohn’s disease patients showed that ulceration developed most readily at the site of the greatest density of end-arterioles, namely along the mesenteric border \{Anthony, Dhillon, et al. 1997\}. Ulcers in rats with indomethacin-induced colitis have been found to have lesions in a similar anatomical distribution \{Anthony, Pounder, et al. 2000\}, suggesting that some areas of the gut are particularly sensitive to inflammatory damage on account of their blood supply. These findings suggest that the processes of ischaemia and vasculitis are important in IBD pathogenesis and several theories exist as to how these processes might arise.

#### 1.8.2 Measles virus

Measles virus has been suggested as an aetiological agent in IBD, since it is known to infect the gut and produce a vasculitis \{Wakefield, Ekbom, et al. 1995\}. Measles viral genomic products have been detected in mucosal biopsy samples of
patients with active IBD by immunogold electron microscopy {Lewin, Dhillon, et al. 1995}. These findings are controversial, since others have been unable to identify measles viral products, despite using similar techniques {Afzal, Armitage, et al. 1998}. Factors which might enhance latent measles virus infection in the gut, such as measles vaccination {Thompson, Montgomery, et al. 1995} and infection with other paramyxoviruses (e.g. mumps) within 12 months of measles infection, have been suggested as increasing the risk of developing IBD {Montgomery, Morris, et al. 1999}.

1.8.3 Autoimmune theories

Anti-neutrophil cytoplasmic antibodies (ANCA) have been found to be associated with vasculitic conditions such as Wegener’s granulomatosis. The prevalence of p-ANCA positivity has been noted to be increased in IBD patients, particularly those with colitis {Cambridge, Rampton, et al. 1992}. Furthermore a discrete disease phenotype has been linked to p-ANCA positivity, which is relatively resistant to medical therapy {Hirv, Seyfarth, et al. 1999}. Other workers have reported an association between p-ANCA positive IBD patients and primary sclerosing cholangitis {Vidrich, Lee, et al. 1995}. It has been suggested that ulcerative colitis is itself an autoimmune disease and exhaustive searches for the antigen responsible for generating disease have been made. Tropomyosin, a smooth muscle antigen, has been proposed as a putative trigger {Das, Dasgupta, et al. 1993} but others have been unable to replicate these findings {Hamilton, Bradley, et al. 1995}.

1.8.4 Thrombophilia and IBD

A wealth of case reports exist to support the observation that patients with active IBD have an increased risk of developing venous thromboses. Proponents of the vasculitis hypothesis have suggested that IBD represents a similar process to systemic lupus erythematosis, a condition in which anti-cardiolipin antibodies, a heightened acute inflammatory state and the presence of vasculitis all contribute to an increased risk of arterio-venous thromboses {Hudson, Chitolie, et al. 1996}.
1.8.4.1 Genetically-acquired thrombophilia

Factor V Leiden mutation results in activated protein-C resistance, which in turn produces an increased risk of thrombosis, more marked in homozygotes than heterozygotes. The prevalence of this pro-thrombotic mutation has been recorded in several population-based studies of IBD patients, with varying results {Liebman, Kashani, et al. 1998}{Vecchi, Sacchi, et al. 2000}. Whether Factor V Leiden mutation is more prevalent in IBD patients remains speculative. Although the mutation has been recognised to be associated with splanchnic thrombosis in IBD, large, adequately powered, prospective studies are awaited. Other pro-thrombotic genes studied in IBD include anti-thrombin III deficiency, hyperhomocysteinaemia and the prothrombin gene mutation. None of these gene mutations in isolation have been shown convincingly to be positively associated with IBD.

1.8.4.2 Platelets

Thrombocytosis is a common finding in patients with active IBD and is thought to be a manifestation of the acute inflammatory response {Collins, Cahill, et al. 1994}. The factors which trigger thrombocytosis include IL-1 and IL-8 {Schaufelberger, Uhr, et al. 1994}. Platelets of IBD patients have been shown to have a relatively increased aggregation potential, which is related to disease activity {Collins, Cahill, et al. 1994}. Furthermore, drugs which inhibit prostaglandin-mediated platelet aggregation, such as Ridogrel, have been shown to be of therapeutic benefit in IBD {Carty, Macey, et al. 2000}. Although it remains unclear whether increased platelet aggregation is a primary or secondary phenomenon in IBD, the beneficial effects of its inhibition add weight to the hypothesis that IBD is primarily a vascular disease.

1.8.4.3 Reproductive hormones

The oral contraceptive pill has been reported to be associated with a higher relapse rate of Crohn’s disease {Timmer, Sutherland, et al. 1998}, although this finding has been disputed {Cosnes, Carbonnel, et al. 1999}. Given that oral contraceptives are recognised to be pro-coagulant, some have suggested that the possible association in Crohn’s disease supports the hypothesis that micro-infarction is the prime aetiological mechanism in IBD {Wakefield, Sawyerr, et al. 1991}. Peri-
Menstrual exacerbation of IBD is also well-recognised in female patients and presumably relates to fluctuating oestrogen and progesterone levels {Kane, Sable, et al. 1998}.

1.8.4.4 Anti-coagulant factors and IBD

There is relatively strong evidence to suggest that factors which inhibit coagulation may be protective against IBD. Firstly, the prevalence of IBD has been found to be significantly lower in patients with inherited bleeding disorders such as haemophilia and von Willibrand’s disease {Thompson, Wakefield, et al. 1995}. Secondly, heparin has been shown to be of therapeutic benefit in IBD, particularly ulcerative colitis {Ang, Mahmud, et al. 2000}{Evans, Wong, et al. 1997}. It has been proposed that heparin’s anti-inflammatory properties are due to its ability to restore the activity of mucosal anti-inflammatory growth factors (basic fibroblast growth factor) by replacing heparan sulphate proteoglycans (e.g. syndecan-1) known to be depleted in IBD {Day, Ilyas, et al. 1999}. Interestingly, warfarin does not seem to share this effect {Gaffney, Doyle, et al. 1995}, suggesting that factors other than inhibition of coagulation are important in the efficacy of heparin.
Chapter 2
Immunological factors and IBD pathogenesis

T cells
This section outlines the rationale for considering the importance of T cells in generating the inflammatory processes of IBD. Their interaction with other mediators, including cytokines, nuclear transcription factors and extra-cellular matrix constituents, will be discussed. The factors which regulate lymphocyte interaction and migration through vascular endothelium will be described in a subsequent section.

2.1 Evidence that T cells are important effector cells in IBD

2.1.1 Evidence from studies of mucosal T cells
By at least 1951, histopathologists had established that the inflammatory infiltrate present in IBD-affected mucosa was largely composed of lymphocytes and macrophages, the presence of granulomata being specific to Crohn’s disease {Rappaport, Burgoyne, et al. 1951}. Early immunohistochemical studies of colonic mucosa from IBD patients probably provided the first evidence that T cells were key mediators in IBD pathogenesis, particularly Crohn’s disease. By distinguishing between B and T cells, and later T-helper (CD4+) and T-suppressor / cytotoxic cells (CD8+), researchers were able to determine that much of the lamina propria infiltrate of IBD comprised of these cell subsets {Selby, Janossy, et al. 1984}.

Cell isolation methods, such as fluorescence-activated cell-sorter analysis (FACS) and flow cytometry, have been used to demonstrate that mucosal T cells highly express activation markers such as the 4F2 antigen, the T9 transferrin receptor (CD-1), HLA-DR and the IL-2 receptor {Pallone, Fais, et al. 1987}. Functional studies of mucosal T cells have been used to show that they exhibit enhanced proliferative responses to bacterial antigens, {Fiocchi, Battisto, et al. 1981} although suppressor cell activity shows wide variability between studies {Fiocchi, Youngman, et al. 1983}. Mucosal T cells in ulcerative colitis particularly, seem to have abrogated IL-2-induced cytotoxicity compared to mucosal T cells from
patients with Crohn’s disease {Kusugami, Youngman, et al. 1989}. More recent functional studies of mucosal T cells in IBD have suggested that they are more resistant to apoptosis in Crohn’s disease than in ulcerative colitis {Ina, Itoh, et al. 1999}.

2.1.2 Evidence from studies of peripheral blood

Initial studies of peripheral blood of patients with IBD suggested that the proportion of activated T cells was increased in patients with active disease {Fais, Pallone, et al. 1985} and that expression of the major histocompatibility complex (MHC) class 1, or HLA-DR, was also increased on both the peripheral blood and mucosal lymphocytes {Mahida, Wu, et al. 1988}. Much early immunological work focussed on whether natural killer (NK) cells {Ginsburg, Dambrauskas, et al. 1983} and suppressor T cells {Hodgson, Wands, et al. 1978} were in some way defective in IBD patients, with different methods of analysis providing conflicting results {MacDermott, Bragdon, et al. 1984}.

More recently, the advent of immunohistochemistry and FACS analysis has provoked further research in this area, once again with conflicting findings. Firstly, studies of macroscopically normal-looking colonic tissue have suggested that whereas in Crohn’s disease mucosal NK cells appear to be increased in number, the reverse is true in ulcerative colitis {van Tol, Verspaget, et al. 1992}. In contrast, studies of peripheral blood lymphocytes in both types of IBD suggest that NK cell activity of these cells is reduced {Giacomelli, Passacantando, et al. 1999}. FACS analyses of peripheral blood lymphocytes have also suggested that the proportion of activated memory T cells (CD45RO+) is increased in the peripheral blood of patients with active IBD compared to controls {Roman, Manzano, et al. 1996}. Furthermore, Crohn’s disease patients have increased proportions of circulating cells expressing the IL-2 receptor (CD25+) {Choy, Walker-Smith, et al. 1990}. Both of these findings suggest that a greater proportion of lymphocytes in IBD are in an activated state compared to those unaffected by the disease, CD45 being a lymphocyte tyrosine kinase involved in T cell activation {Koretzky, Picus, et al. 1990}, and IL-2 a pro-inflammatory cytokine.
2.1.3 Evidence from studies of T cell receptor subtypes

Studies of the Vβ region of the T cell receptor in patients with Crohn’s disease and ulcerative colitis have shown that clonal expansion of CD4 cells expressing specific Vβ chains exists {Probert, Chott, et al. 1996}, suggesting that T cells respond to a range of IBD-specific antigens. Other workers have demonstrated similar findings in Crohn’s disease patients with coexistent arthropathies {Lugering, Kucharzik, et al. 1996}. The precise antigens which trigger activated T cells have not been identified, although endogenous bacterial antigens have been proposed {Duchmann, May, et al. 1999}.

The vast majority of mucosal T cells express the αβ-type T cell receptor (TCR), but T cells which express the less common γδ-type TCR may also be important in IBD. Increased proportions of these TCRγδ+ cells have been found in the circulation of Crohn’s patients in particular {Soderstrom, Bucht, et al. 1996}. Mucosal proportions of these cells have been found to be generally reduced in IBD mucosa {Fukushima, Masuda, et al. 1991}, although others have reported raised proportions in lymphoid aggregates in the colonic mucosa of ulcerative colitis patients {Yeung, Melgar, et al. 2000}. The precise role of TCRγδ+ cells in IBD is not yet clear, although animal studies suggest that they may protect against inflammation, since TCRγδ+ cell-deficient mice have exaggerated responses to intestinal pathogens {Roberts, Smith, et al. 1996}.

2.1.4 Evidence for T cell importance from animal models of IBD

Earlier animal studies relied mostly on chemically-induced colitis or spontaneous development of colitis in animals in captivity, such as the cotton-top tamarin {Madara, Podolsky, et al. 1985}. Adoptive transfer of certain T cell subtypes has also been found to result in colitis, such as CD45Rb\textsuperscript{high}, a form of memory T cell {Powrie & Leach 1995}. It was recognised subsequently that a wide variety of different genetic manipulations could result in a disease similar to IBD in mice, although most commonly this took the form of a colitis, rather than ileal inflammation. One of the earliest genetic ‘knockout’ models of IBD to suggest that T cells were central to its pathogenesis was that of the colitic TCRα-deficient mouse {Mombaerts, Mizoguchi, et al. 1993}. Early appendicectomy has been found to be protective in such animals, highlighting the importance of gut
lymphoid tissue in mediating inflammation {Mizoguchi, Mizoguchi, et al. 1996}. Many other transgenic and knockout rodent models of IBD, based largely on manipulations of cytokine genes, will be discussed in a later section.

2.1.5 Evidence for T cell importance from clinical studies
Crohn’s disease patients treated with monoclonal antibodies to CD4 cells have been found to experience symptomatic relief {Stronkhorst, Radema, et al. 1997}, highlighting the importance of T cells in IBD. In addition, some studies suggest that IBD patients with HIV infection develop improvement of their symptoms once their CD4 count drops {Sharpstone, Duggal, et al. 1996} although this has been disputed {Christ, Sieber, et al. 1996} {Louis, Moutschen, et al. 1997}.

2.2 The relationship between T cells and other mediators

2.2.1 Cytokines: an overview
Cytokines are regulatory proteins released mostly by activated T or B lymphocytes in response to a stimulus. They act as messengers to alter the phenotype and activation state of other immune effector cells and also trigger them to produce other cytokines. It has been hypothesised that IBD and other chronic inflammatory conditions represent an imbalance of cytokine production. Certain cytokines appear to act predominantly as pro-inflammatory mediators (e.g. TNF-α, IL-1 and IL-6), whereas others appear to protect or down-regulate inflammatory responses of other effector cells (interleukins 2, 4, 10 and 11). It has been suggested that Crohn’s disease represents over-activation of ‘Th1-type’ or pro-inflammatory cytokines, with down-regulation of the ‘Th-2 type’ anti-inflammatory cytokines {Niessner & Volk 1995}. This hypothesis stems largely from the results of genetic ‘knockout’ mouse studies, such as IL-2- or IL-10-deficient mice, which develop colitis provided that they are not reared in a germ-free environment {Sadlack, Merz, et al. 1993} {Kuhn, Lohler, et al. 1993}. Other genetic ‘knockout’ mouse models have been described in section 2.1.4. More recently, mice which over-produce TNFα due to deletion of genes controlling its synthesis, develop patchy ileo-colonic inflammation which is similar to Crohn’s disease {Pizarro, Arseneau, et al. 2000}. 
2.2.2 Pro-inflammatory cytokines

Studies in man suggest that pro-inflammatory cytokines are up-regulated in IBD. Initial studies of cytokines in IBD generally relied on assaying either serum samples, tissue supernatants or homogenates of inflamed tissue compared to normal controls. Such studies provided somewhat conflicting results, probably due to variations in methodology and lack of understanding of factors affecting cytokine stability in vitro {Fiocchi, Fukushima, et al. 1996}. More recently, quantitative methods of assaying cytokine production of individual cells (e.g. ELISPOT analysis) have provided more robust data and have enhanced understanding of the role of cytokines in IBD {MacDonald, Hutchings, et al. 1990}. It is now known that TNFα production is enhanced throughout the GI tract of Crohn’s patients, and not just at the site of mucosal lesions, in addition to other pro-inflammatory cytokines (IL-1β and IL-6) {Reimund, Wittersheim, et al. 1996}.

IL-1 is a pro-inflammatory cytokine also thought to be important in IBD. It is associated with the initiation and amplification of inflammation in some animal models of colitis and the administration of IL-1 receptor antagonist (IL-1Ra) has been found to prevent the development of experimental colitis in the rabbit {Cominelli, Nast, et al. 1990}. Although an imbalance between IL-1 and IL-1Ra appears to exist in IBD {Casini-Raggi, Kam, et al. 1995}, clinical studies of IL-1Ra have yet to be conducted.

2.2.3 The role of TNFα in IBD; evidence from anti-TNFα therapies

Anti-TNFα treatment is the first successful targeted immunotherapy to be widely utilised in IBD and probably represents the greatest advance in its treatment since the introduction of azathioprine as steroid-sparing agent approximately 30 years ago {Brown & Achkar 1970}. Its success strongly supports the argument that TNFα is a key cytokine mediator in IBD pathogenesis. The hypothesis that blockade of TNFα might be of therapeutic benefit in IBD stemmed from early studies of cytokine expression in IBD patients, in whom it was noted that TNFα was increased in both inflamed colonic mucosa and stool {Murch, Braegger, et al. 1993} {Braegger, Nicholls, et al. 1992}. Animal studies, notably of rats with colitis induced by rectal 2-4-6-trinitrobenzene sulphonic acid (TNBS), confirmed
that treatment with a single infusion of CA2, a chimeric monoclonal antibody to 
TNFα, resulted in resolution of colitis and improved animal survival (Neurath, 
Fuss, et al. 1997). CA2, better known as infliximab, is a chimeric monoclonal 
antibody to TNFα, which consists of approximately 25% murine protein and 
which fixes complement as a result of being an IgG1 antibody. It binds to both 
free and membrane-bound TNFα, resulting in lysis of those cells to which TNFα 
is bound.

2.2.3.1 Clinical trials of infliximab
The first successful clinical trials of TNFα blockade were in patients with 
rheumatoid arthritis, a chronic inflammatory condition which bears some 
mechanistic similarities to IBD (Elliott, Maini, et al. 1994). Subsequently, an 
open label study demonstrated that a single infusion of 10mg/kg TNFα antibody 
(CA2/infliximab) produced a significant reduction in disease activity in 10 patients 
with active Crohn’s colitis for at least four weeks (van Dullemen, van Deventer, 
et al. 1995). A pivotal randomised double-blind, placebo-controlled study 
followed in 108 patients with moderate to severely active Crohn’s disease 
(Targan, Hanauer, et al. 1997). This study showed a remission rate of 39% and 
response rate (fall in Crohn’s disease activity index [CDAI] of >70 points) of 67% 
at four weeks post-infusion. Further studies have demonstrated that infliximab is 
of benefit in fistulising Crohn’s disease (Present, Rutgeerts, et al. 1999) and early 
studies suggest that remission may be maintained in active Crohn’s disease for up 
to 12 months with repeat infusions every two months (Rutgeerts, D’haens, et al. 
1999).

Despite being a highly effective therapy, questions of infliximab’s safety exist, 
since at least 13% of patients develop human anti-chimeric antibodies (HACA) 
after a second infusion. If such patients receive further infusions after a treatment 
‘gap’ of at least six months, approximately 10% will have a serum sickness-type 
reaction which may be serious (Hanauer 1999). In addition, if a patient develops 
high titre HACA, the drug’s efficacy is irreversibly lost. Concurrent azathioprine 
use appears to enhance the drug’s efficacy and reduces the likelihood of 
developing HACA. Other safety issues raised by post-marketing surveillance 
include an increased risk of patients developing tuberculosis post-infliximab, the
development of anti-double stranded DNA antibodies and a suggested link with the development of lymphoma.

2.2.3.2 Clinical trials of CDP571

CDP571 is another humanised chimeric antibody to TNFα, which is hoped to be less immunogenic than infliximab, since its murine component is just 5% and being an IgG4 antibody, does not fix complement. CDP571 has been found to be of benefit in both mild to moderately active Crohn’s disease in a double-blind, placebo-controlled study {Stack, Mann, et al. 1997} and also in an open study of ulcerative colitis patients {Evans, Clarke, et al. 1997}. Two randomised placebo-controlled studies of CDP571 have been published, both of which suggest that it may be of benefit in both moderate to severely active and remission maintenance in Crohn’s disease {Sandborn, Targan, et al. 2000}{Feagan, Sandborn, et al. 2000}.

2.2.3.3 Etanercept

One method of reducing the immunogenicity of anti-TNFα therapies has been to design a ‘small molecule’ treatment, etanercept, which consists of a recombinant soluble version of the p75 TNF receptor protein linked to the Fc portion of human IgG1. Etanercept has been found to be efficacious in rheumatoid arthritis {Moreland, Schiff, et al. 1999} and in a small open study of patients with active Crohn’s disease {D’haens, Swijsen, et al. 2000}. A larger randomised placebo-controlled trial of etanercept has not shown an efficacy benefit in patients with active Crohn’s disease, however {Sandborn, Hanauer, et al. 2001}.

2.2.3.4 Other anti-TNFα therapies in IBD

Thalidomide has been examined as a possible anti-TNFα treatment, since it directly inhibits TNFα synthesis. Early studies have suggested modest benefit in patients with active Crohn’s disease {Ehrenpreis, Kane, et al. 1999} but the drug’s use may be limited by its unwanted side effects, namely, teratogenicity, drowsiness and peripheral neuropathy. Secondly, pentoxifylline has been found to inhibit release of TNFα and other pro-inflammatory cytokines from human peripheral blood monocytes in vitro {Reimund, Dumont, et al. 1997}. Studies in animal models of IBD have suggested that pentoxifylline may be a promising
treatment for IBD, by abrogating colitis {Murthy, Cooper, et al. 1999} {Peterson & Davey 1997}. To date, however, clinical studies in Crohn’s disease patients have shown no significant therapeutic benefit attributable to pentoxifylline {Bauditz, Haemling, et al. 1997}.

2.2.4 Evidence from clinical trials of the importance of ‘anti-inflammatory’ cytokines in IBD

2.2.4.1 Interleukin 10

Studies of IL-10-deficient colitic mice reared in a ‘germ-inclusive’ environment led to the hypothesis that IBD patients may be deficient in IL-10 and that IL-10 itself may be an effective treatment for IBD. Clinical trials of recombinant human IL-10 (rhuIL-10) have been somewhat disappointing, however. Initial pilot open studies of repeated intravenous doses of rhuIL-10 suggested therapeutic benefit in patients with active Crohn’s disease {Van Deventer, Elson, et al. 1997} and ulcerative colitis {Schreiber, Fedorak, et al. 1998}. A larger multicentre study of multiple subcutaneous doses of rhuIL-10 in patients with active Crohn’s disease did not demonstrate a significant treatment benefit when compared with placebo {Schreiber, Fedorak, et al. 2000} although the change from intravenous to subcutaneous preparations may have affected efficacy.

2.2.4.2 Interleukin 11

Studies in the HLA-B27 rat model of colitis have suggested that IL-11 may protect against gut inflammation {Peterson, Wang, et al. 1998}. A small safety study of recombinant IL-11 has been conducted in patients with active Crohn’s disease {Sands, Bank, et al. 1999}, but results of randomised placebo-controlled studies are awaited.

2.2.5 Nuclear transcription factors

Nuclear factor kappa B (NFκB) acts by controlling the nuclear transcription of various immune mediators, in particular pro-inflammatory cytokines such as TNFα. NFκB has also been found to be up-regulated in the inflamed mucosa of Crohn’s disease patients {Ellis, Goodlad, et al. 1998}. Specific blockade of NFκB in vitro has been found to reduce leucocyte release of IL-2 and TNFα. Targeted blockade of NFκB has yet to be performed in IBD patients, although preliminary
studies of an antisense nucleotide to NFκB in mice with dextran-sodium sulphate-
induced colitis (DSS) suggest that it may reduce gut inflammation {Murano,
Maemura, et al. 2000}. Furthermore, antisense oligonucleotide to the p65 subunit
of NFκB has been found to markedly reduce release of IL-1, IL-6 and TNFα in
vitro from macrophages of patients with active Crohn’s disease {Neurath,
Pettersson, et al. 1996}. Interestingly, some well-established treatments for IBD
demonstrate NFκB inhibition in vitro, such as corticosteroids and sulphasalazine
{Ardite, Panes, et al. 1998}, which may partly explain their efficacy in vivo.

Less is understood about the role of chemokines in IBD. One example is CCR5, a
chemokine which is thought to play an important role in regulating T cell
activation, although transcription has not been found to be altered in IBD patients
{Martin, Heinzlmann, et al. 2001}.

2.2.6 Extra-cellular matrix components

In addition to factors released by leucocytes, other factors impact on T cells which
may affect the pathogenesis of IBD. For example, much work has centred on
factors which reduce the integrity of the barrier function of the gut mucosa.
Firstly, immunohistochemical studies of gut mucosal and submucosal biopsies of
paediatric IBD patients suggest that extra-cellular matrix proteins such as
sulphated glycosaminoglycans (GAGs) are markedly disrupted {Murch,
MacDonald, et al. 1993}. Furthermore, treatment of children with distal colitis
with topical enema preparations of N-acetyl-glucosamine improves inflammation
micro- and macroscopically, by restoring sulphated GAG synthesis {Salvatore,
Heuschkel, et al. 2000}. Results of randomised double-blind placebo-controlled
trials are awaited. Secondly, mucin-depletion of colonic mucosa is a characteristic
histological feature of ulcerative colitis. In vitro studies of inflamed mucosa of
such patients have shown that mucin secretion can be increased by agents known
to be beneficial in ulcerative colitis, such as corticosteroids, nicotine and butyrate
{Finnie, Dwarakanath, et al. 1995} {Finnie, Campbell, et al. 1996}. The
relationship between T cells and mucin depletion remains to be elucidated.

Matrix metallo-proteases are endogenous enzymes found in the gut mucosal extra-
cellular matrix which are thought to enhance mucosal inflammation and
disruption. Their activity has been found to be increased in IBD tissue and in human foetal gut organ culture systems. Their activity may be regulated by activated T cells {Pender, Tickle, et al. 1997} and inhibited by IL-10 {Pender, Breese, et al. 1998}. Stromelysin-1 seems to have a more important role in generating inflammation than tissue inhibitor of metalloprotease (TIMP-1) {Heuschkel, MacDonald, et al. 2000}. Inhibitors of matrix metalloproteases, such as marimastat, appear to reduce gut inflammation in animal models of disease {Sykes, Bhogal, et al. 1999} and may yet have a role in IBD itself.

2.2.7 Eicosanoids

Eicosanoid is the collective term for products derived from the breakdown of membrane phospholipids to arachidonic acid and formation of leukotrienes, prostaglandins and thromboxanes. The role of these products in IBD is not clear, although initial studies of prostaglandins found them to be elevated in ulcerative colitis mucosa {Rampton, Sladen, et al. 1980} and levels reduced by sulphasalazine treatment {Rachmilewitz, Stamler, et al. 1995}. Other eicosanoids, including thromboxanes, prostacyclines and leukotriene B4 have been found to be elevated in both ulcerative colitis and Crohn's disease {Gertner, Rampton, et al. 1994} {Ligumsky, Karmeli, et al. 1981}. Like cytokines, they appear to have both a pro-inflammatory and protective effect against inflammation, although how they interact with T cells is unclear. That eicosanoids may be cytoprotective in IBD is suggested by the exacerbatory effect of non-steroidal anti-inflammatory drugs (NSAIDS) on IBD, since these drugs inhibit eicosanoid formation by inhibiting cyclo-oxygenase (COX) activity. Animal and in vitro studies would suggest that even NSAIDS with selective COX-2 inhibitory effects may exacerbate IBD {McCartney, Mitchell, et al. 1999} {Reuter, Asfaha, et al. 1996}. Conversely, thromboxanes may be pro-inflammatory in IBD, since selective inhibition by picotamide or ridogrel has been found to result in reduced evidence of inflammation in IBD mucosa in vitro {Carty, MacEy, et al. 2000} {Collins, Benson, et al. 1996}.

2.2.8 Reactive oxygen and nitrogen metabolites

Mucosal infiltration by neutrophils is well-recognised as being part of the inflammatory process of Crohn's disease and ulcerative colitis, although their
mechanism of interaction with other inflammatory cells such as T cells is undetermined. Neutrophils have been found to be potent sources of reactive oxygen metabolites (ROMs) and nitrogen metabolites and there is evidence to suggest that both are important mediators of inflammation in IBD. Firstly, chemiluminescence studies of IBD-affected gut mucosa suggest that the presence of ROMs is directly related to disease activity (Simmonds, Allen, et al. 1992). Furthermore, nitric oxide generation and nitric oxide synthase activity appears to be increased in IBD enteric tissue (Boughton-Smith, Evans, et al. 1993), whereas nitric oxide synthase is not present in normal colonic epithelium (Singer, Kawka, et al. 1996). 5-aminosalicylates appear to have anti-oxidant action on colonic tissue in vitro, suggesting a mechanism for their therapeutic effect (Simmonds, Millar, et al. 1999).

2.2.9 Growth factors
Transforming growth factor-beta (TGFβ) has been proposed to be responsible for promoting healing of inflamed mucosa in IBD, whereas TGFα has a more proliferative action. Both are increased in concentration in active compared to normal mucosa in IBD patients (Babyatsky, Rossiter, et al. 1996). Studies of paediatric IBD patients suggest that enrichment of polymeric diets with TGFβ2 may improve the response to this alternative treatment to corticosteroids for active disease (Fell, Paintin, et al. 2000). How TGFβ might suppress T cell-mediated inflammation is unclear.

2.3 Leucocyte adhesion and IBD

2.3.1 Introduction
A wide variety of glycoprotein cellular adhesion molecules are recognised to be important mediators of leucocyte trafficking to the gut. Many of these mechanisms have been studied in animal models of colitis and patients with IBD. Increased adhesiveness of leucocytes extracted from chronically inflamed but not normal intestine of IBD patients has been demonstrated (Binion, West, et al. 1998), suggesting that increased leucocyte binding in IBD patients is an acquired phenomenon which might serve as a target for future therapis. The role of cellular adhesion molecules in IBD is discussed overleaf.
2.3.2 Selectins and leucocyte rolling

The first stage of leucocyte migration through gut endothelium is thought to involve a process of rolling, which is controlled largely by selectins present on the cell surface (Figure 1, page 54). These transmembrane glycoprotein receptors, designated E-, L-, and P-selectin, all recognise ligands that are decorated with the fucosylated sialyl-Lewis x tetrasaccharide, the docking site for which has been mapped onto the crystal structure of E-selectin {Steegmaier, Levinovitz, et al. 1995}. L-selectin, alternatively known as CD62L, LAM-1, LECAM-1 or ME-14 Ag, is the predominant leucocyte selectin, being expressed mainly on B cells, naïve T cells, circulating monocytes and neutrophils. Rolling is mediated by the interaction of L-selectin with reciprocal endothelial cell selectins, namely P-selectin (CD62P, PADGEM, or GMP-140), which is also expressed on platelets, and E-selectin (CD62E or ELAM-1) which is present on activated endothelial cells only. In the gut, L-selectin may also interact with the endothelial mucosal addressin cellular adhesion molecule (MAdCAM-1) and other putative selectin ligands, including endothelial glycoproteins CD34 and Gly-CAM-1.

Intravital microscopic studies in mice have shown that reduction in selectin interactions, either by monoclonal antibody blockade {Ley, Bullard, et al. 1995} or gene deletion, reduces the ability of lymphocytes to roll on mesenteric vascular endothelial cells {Kunkel & Ley 1996}. Additionally, monoclonal antibodies to E-selectin reduce human neutrophil adhesion to endothelial cell lines in vitro {Kishimoto, Warnock, et al. 1991}, and immunohistochemical analyses of IBD-affected colonic tissue show up-regulation of selectins compared with that of healthy subjects {Koizumi, King, et al. 1992}. More recently, radiolabelled E-selectin has been used to image IBD-affected bowel by scintigraphy and such scans correlate well with the results of colonoscopy and barium studies {Bhatti, Chapman, et al. 1998}. This study and others also report a positive correlation between disease activity in IBD and serum concentrations of soluble, presumed ‘shed’, E- and P-selectins {Patel, Pall, et al. 1995}, although other studies have not confirmed this relationship {Goke, Hoffmann, et al. 1997}. Finally, a recombinant human P-selectin glycoprotein ligand has been found to reduce the intestinal lesions of rats with experimental colitis {Albert, Patel, et al. 1998}, whilst an orally-active E-selectin inhibitor has been found to be efficacious in
colitic macaques {Jurgensen, Daluge, et al. 2000}. To date, there have been no studies of selectin inhibition in IBD patients.

2.3.3 Integrins, leucocyte adherence and emigration

Following rolling, integrins and their reciprocal ligands control leucocyte adherence to, and emigration through, vascular endothelium. These heterodimeric transmembrane glycoproteins are expressed on the surface of most leucocytes and consist of α and β sub-units. At least 18α and 8β subunits have been described, of which the β2 subfamily (αLβ2/ CD11aCD18/ LFA-1; αMβ2/ CD11Bcd18/ Mac-1; αxβ2/ CD11CCD18/ p150), the α4 subfamily (α4β1/ CD49d/ VLA-4; α4β7) and αEβ7 integrins are thought to be particularly relevant in IBD.

2.3.4 Beta-2 integrins

The expression of β2 integrins has been shown to be increased on gut mucosal mononuclear cells of rodents with experimental colitis {Palmen, Dieleman, et al. 1995} and patients with IBD {Nakamura, Ohtani, et al. 1993}. Additionally, anti-β2 monoclonal antibody treatment of rats with TNBS-induced colitis resulted in reduced colonic ulceration and leucocyte infiltration {Palmen, Dijkstra, et al. 1995}. β2 integrins interact predominantly with endothelial cells which express the intercellular adhesion molecule (ICAM-1/ CD54a; ICAM-2/ CD 102) ligand subfamily. Patients with IBD have been shown to have both raised serum soluble ICAM-1 concentrations {Nielsen, Langholz, et al. 1994} and increased endothelial cell expression of ICAM-1 in inflamed gut compared with healthy subjects {Koizumi, King, et al. 1992}. Serum findings have been disputed by other workers {Goke, Hoffmann, et al. 1997}.

Intravenous ICAM-1 antisense oligonucleotide has been shown to reduce DSS-induced colitis in mice, by selective inhibition of cytokine-induced ICAM-1 expression {Bennett, Kornbrust, et al. 1997}. A clinical trial has also shown that intravenous ICAM-1 antisense oligonucleotide (ISIS 2302) affects the course of active Crohn's disease {Yacyshyn, Bowen-Yacyshyn, et al. 1998}. In the latter pilot double-blind, placebo-controlled study, 47% (7/15 patients) of patients who received ISIS 2302 were in remission (CDAI<150 points) at the four week endpoint, compared with 20% (1/15) in the placebo group, although this difference is
not significant. Furthermore, corticosteroid use was significantly lower and colonic mucosal expression of ICAM-1 was reduced in those who received the active drug {Bowen-Yacyshyn, Shanahan, et al. 1998}. A larger multicentre randomised, placebo-controlled trial of this treatment was subsequently conducted in USA, Canada and Europe in patients with active steroid-dependent Crohn’s disease. The results of this trial showed no efficacy benefit of ISIS 2302 compared to placebo {Yacyshyn, Chey, et al. 2000}.

Somewhat paradoxically, a rare inherited β2 integrin deficiency (CD11/CD18 deficiency) has been described in association with chronic enterocolitis, which is macroscopically indistinguishable from Crohn’s disease {D'Agata, Paradis, et al. 1996}. This suggests that selective β2 integrin blockade alone may not be sufficient to induce remission of IBD.

### 2.3.5 Animal studies of alpha-4 integrins

The role of α4 integrins, which exist on leucocytes mainly in association with β1 or β7 integrins, has been studied extensively. Both α4β1 and α4β7 integrins interact with reciprocal endothelial ligands fibronectin and vascular cellular adhesion molecule-1 (VCAM-1/CD106), but in vitro studies suggest that only α4β7 has a high affinity for MAdCAM-1, which is expressed mainly on Peyer’s patch endothelium {Erle, Briskin, et al. 1994}. The hypothesis that α4β7 integrins are essential for leucocyte homing to gut mucosa is based largely on evidence from animal studies. Studies of transgenic mice have shown that α4 null chimeric mice are unable to recruit T cells to Peyer’s patches {Arroyo, Yang, et al. 1996}. Furthermore, Peyer’s patches are absent in β7 deficient animals {Wagner, Lohler, et al. 1996}, suggesting that both α4 and β7 integrins are essential for the formation of gut-associated lymphoid tissue.

Studies of other animal models of colitis, such as the cotton-top tamarin and CD45RB^{high}-reconstituted SCID mouse, have shown that intravenous antibodies to α4 {Podolsky, Lobb, et al. 1993}, α4β7 {Hesterberg, Winsor-Hines, et al. 1996}, or β7 integrin {Picarella, Hurlbut, et al. 1997} significantly attenuate colonic inflammation. The role of the α4 endothelial ligand, VCAM-1, in gut
inflammation is less clear. Studies of β2-integrin deficient mice have shown that VCAM-1 may facilitate homing of α4+ leucocytes to gut mucosal lymphoid tissue in addition to MAdCAM-1 \cite{Berlin-Rufenach1999}. Furthermore, intravital studies in rats have shown that VCAM-1 is up-regulated on colonic venous endothelial cells in TNBS-induced colitis \cite{Sans1999}, suggesting that it too has a role in inflammation.

2.3.6 Clinical studies of alpha-4 integrins

Alpha-4 integrins appear to be critical to enable leucocyte-homing to the human gut \cite{Hamann1994}, particularly when combined with β7 integrins, although conflicting results have been obtained from FACS analyses of α4β7 expression by mucosal and peripheral blood lymphocytes in IBD patients. For example, Crohn's disease patients appear to have increased expression of α4β7 integrin on lamina propria lymphocytes (LPL) \cite{Meenan1997}, although the expression on peripheral blood lymphocytes is not elevated, irrespective of disease activity state \cite{Dhiman1998}.

Immunohistochemical studies have demonstrated that MAdCAM-1, the endothelial α4β7 ligand, is more readily detected in active Crohn's disease and ulcerative colitis gut mucosa than in that of healthy controls, but the expression of VCAM-1, the α4β1 ligand, appears to be reduced or absent in IBD-affected tissue \cite{Farstad1997}. Furthermore, FACS analysis of lymphocytes extracted from the thoracic duct at oesophagectomy shows them to have higher memory T cell expression of α4β7 relative to peripheral blood, implying that this is a major trafficking route for this lymphocyte subset \cite{Lemaire1997}. The α4β1 integrin is thought to be important in lymphocyte trafficking to peripheral lymph nodes and lymphocyte activation. Its significance in IBD is not yet clear, although its expression has been shown to be increased in LPLs taken from ulcerative colitis patients compared with the expression of other β1 integrins \cite{Künne1997}.

Natalizumab is a humanised monoclonal antibody to α4 integrin. Its action forms the basis for much of this thesis and relevant pre-clinical work is discussed in
section 2.4. Act-1 is a different humanised monoclonal antibody to \( \alpha 4 \) integrins, which has been designed to target the \( \alpha 4 \beta 7 \) integrin complex. A pilot randomised placebo-controlled study of this antibody has suggested therapeutic benefit in patients with ulcerative colitis {Feagan, McDonald, et al. 2000}. Ultimately, smaller peptide \( \alpha 4 \) integrin inhibitors or integrin antisense molecules, with less potential to induce neutralising antibody production, are likely to be developed as a more suitable therapy for repeated treatments.

2.3.7 Alpha-E integrins, cadherins and catenins

Lastly, \( \alpha E \beta 7 \) integrin is expressed largely by intestinal epithelial lymphocytes and interacts with intestinal epithelial cadherins and catenins (\( \alpha \), \( \beta \) and \( \gamma \)). The role of this adhesion molecule group is less understood in IBD than it is in other gastrointestinal diseases, such as Barrett’s oesophagus and colonic carcinoma. Nevertheless, transgenic mutant N-cadherin chimeric mice have been found to develop IBD-type intestinal inflammation only in those areas of gut mucosa which express the mutant N-cadherin gene. This suggests that N-cadherin is vital for the maintenance of intact mucosa and that IBD may represent dysregulation of this system {Hermiston & Gordon 1995}. Furthermore E-cadherin, together with the catenins, has been found to be up-regulated in inflamed colonic tissue from IBD patients {Demetter, Cesmali, et al. 1998}, the significance of which is unclear.

2.3.8 Control of adhesion molecule expression: the role of TNF\( \alpha \) and NF\( \kappa B \)

If leucocyte adhesion to vascular endothelium is pivotal in potentiating chronic inflammation in IBD, factors which control expression of adhesion molecules may also represent potential therapeutic targets. One such mediator is TNF\( \alpha \), a pro-inflammatory cytokine which has been found to upregulate adhesion molecule expression on lymphocytes and endothelial expression of P- and E-selectin in mice {Weller, Isenmann, et al. 1992}. At the genetic level, TNF\( \alpha \)-mediated expression of cellular adhesion molecules is regulated by NF\( \kappa B \) {Takeuchi & Baichwal 1995}. Selective blockade of NF\( \kappa B \) by antisense oligonucleotide has been shown to abrogate experimental colitis in rats {Neurath, Pettersson, et al. 1996}, although it is unclear whether reduction of adhesion molecule expression is responsible for this effect. Other cytokines which affect adhesion molecule expression include IL-1 and \( \gamma \)-interferon {Pober, Gimbrone, et al. 1986}. 
2.3.9 Effects of IBD therapies on adhesion molecule expression
Reduction of adhesion molecule expression may contribute to the therapeutic effect of some current IBD therapies. For example, corticosteroids have been shown to reduce P- and E-selectin by murine endothelial cells in the gut (Mori, Horie, et al. 1999) and in vitro work suggests that nicotine may also reduce endothelial E-selectin expression, a finding which may partly explain its protective effect in ulcerative colitis patients (Bhatti, Haskard, et al. 1997). Interestingly, sulphasalazine has been found to have an inhibitory effect on NFκB expression by human colonic epithelial cells in vitro, but it is not known whether this elicits a corresponding reduction in adhesion molecule expression (Wahl, Liptay, et al. 1998).

2.3.10 Summary of leucocyte adhesion mechanisms
The list of ligands and receptors involved in intercellular adhesion continues to expand, although many questions remain unanswered with respect to the precise function of some of these molecules. In particular, the real-time dynamics of adhesion molecule expression and activation is unclear, thus most of the studies described above represent just a snap-shot view of the cell adhesion and migration cascade. Other unexplained issues include the mechanisms by which cellular adhesion molecules are activated in vivo and whether some cells express multiple adhesion molecules in different activity states at once. Studies of other chronic inflammatory processes, such as rheumatoid arthritis, multiple sclerosis, asthma and atherosclerosis suggest that many of the adhesion mechanisms described are ubiquitous; thus emerging anti-adhesion strategies for IBD may have additional therapeutic use in such conditions.
Figure 1

**Leucocyte adhesion**

Leucocyte → Integrins → L-selectin

Leucocyte rolling → Adhesion (α4-mediated or β2-mediated) → Migration

Endothelial cell membrane

**E-selectin** → VCAM-1 or MAdCAM → ICAM-1

**Leucocyte adhesion and natalizumab**

Leucocyte → Integrins → L-selectin → Natalizumab

Leucocyte rolling → Adhesion (α4-mediated) → Migration

Endothelial cell membrane

E-selectin → VCAM-1 or MAdCAM → Natalizumab
2.4 Natalizumab

2.4.1 Background
Natalizumab (Antegren™; Elan Pharmaceuticals, South San Francisco, California U.S.A.) is a recombinant humanised antibody, which has been derived from a murine monoclonal antibody (AN100226m) raised against human α4 integrin. AN100226m was humanised by complementarity determining region-grafting of the hypervariable region of the gene encoding AN100226m onto a human IgG4 framework {Leger, Yednock, et al. 1997}. The resultant antibody contains approximately 5% mouse derived protein only. In vitro, natalizumab has been shown to block the adhesion of human Jurkat α4β1-expressing cells to high density purified recombinant VCAM-1 {Leger, Yednock, et al. 1997} and α4β7-expressing RPMI-8866 cells to recombinant MAdCAM-1, respectively {Yednock 1999}.

2.4.2 Studies of natalizumab in mammals
The effects of natalizumab have been investigated in mice, guinea pigs and cynomolgus monkeys. Toxicity studies in mice and cynomolgus monkeys have shown that a single infusion is safe up to a concentration of 30mg/kg {Athena 1995} {Athena 1996}. Notably, animals experienced a transient, dose-dependent rise in circulating peripheral blood lymphocytes and monocytes but not neutrophils. This parallels the findings in rats and cotton-top tamarins following infusion of monoclonal antibodies to ICAM-1 and α4β7 integrins, respectively {Bennett, Kornbrust, et al. 1997} {Hesterberg, Winsor-Hines, et al. 1996}. Together, these findings suggest that blockade of leucocyte trafficking by interruption of leucocyte-endothelial cell signalling leads to an increase in intravascular leucocyte counts. Alternatively, altered trafficking may stimulate increased release of leucocytes into the intravascular space from extravascular sites, although there are no data to confirm this. The effects of natalizumab have not been tested in the spontaneously colitic cotton-top tamarin, instead detailed studies of its effects on guinea-pigs with experimental allergic encephalomyelitis have been performed {Yednock, Cannon, et al. 1992}. The latter serves as an animal model of multiple sclerosis, in which leucocyte trafficking across neurovascular mucosa is thought to be facilitated by the interaction of leucocyte
α4β1 integrin and endothelial VCAM-1. A single infusion of natalizumab has been shown to produce clearance of leucocytes in the central nervous system of these animals, suggesting that it may be of therapeutic benefit in patients with multiple sclerosis.

2.4.3 Clinical studies of natalizumab

The effect of natalizumab on healthy volunteers was assessed prior to commencing studies in patients with multiple sclerosis or inflammatory bowel disease. Thirty-five healthy males aged 18 to 55 years were exposed to single infusions of between 0.03mg/kg and 3mg/kg {Athena 1996}. No serious adverse effects were noted. Mild adverse events included headache, rhinitis, dizziness, myalgia, dyspepsia and anorexia. One subject was noted to have elevated serum concentrations of alkaline phosphatase and gamma-glutaryl transferase between eight and 36 days post-infusion, but was subsequently found to have gallstones, to which these changes were attributed. Dose-dependent elevations of all peripheral blood leucocyte subsets, except neutrophils, were noted, but volunteers did not appear to be at increased risk of infection. The plasma half-life of a single 3mg/kg dose of natalizumab was 8.7 days. Saturation studies suggested that a single infusion of 3mg/kg was sufficient to produce 80% saturation of circulating leucocyte α4 integrins for up to 22 days post-infusion. Saturation levels dropped below this value when serum concentrations fell below 5μg/ml.

Given the findings in experimental allergic encephalomyelitis, a multicentre, double-blind, placebo-controlled study of two 3mg/kg infusions of natalizumab, administered four weeks apart, was conducted in 72 patients with chronic relapsing multiple sclerosis {Tubridy, Behan, et al. 1999}. The end-points of this study included improvement of disease as estimated by frequency of new plaques seen on magnetic resonance scanning of brain and spinal cord. The study showed a positive efficacy benefit attributable to natalizumab during the first three months post-infusion. Following the results of studies by Podolsky in 1993, using a humanised monoclonal antibody to α4β7 in spontaneously colitic cotton-topped tamarins, it was realised that natalizumab may be of therapeutic benefit in patients with IBD, since it blocks both α4β1 and α4β7 integrins in vitro.
Chapter 3
Clinical trial methods in IBD

This section aims to give an overview of commonly encountered problems in IBD trial design, since much of the practical work of this thesis centres on clinical trials of a new therapy for IBD. Development of selection criteria, methods of assessing efficacy and specific problems associated with concurrent IBD therapies will be discussed in detail.

3.1 Patient selection

3.1.1 Disease definition

Defining the entry criteria for IBD trials may be more difficult than immediately apparent. As with any clinical trial, it is vital to ensure that the precise diagnosis has been confirmed by accepted methods, since the end diagnosis may change in at least 10% of patients within the first year following initial presentation {Moum, Ekbom, et al. 1997}. This also applies to patients whose colitis has been diagnosed on the basis of barium enema and rigid sigmoidoscopic biopsies (common in the U.K. before the 1980s), a method now recognised to be less accurate than colonoscopy {Holmquist, Rudic, et al. 1988}. Diagnostic uncertainty in new IBD patients can usually be overcome by setting a minimum time-limit between the date of diagnosis and the date of study entry (e.g. 6 months or one year). Furthermore, establishing a fixed time-limit prior to trial entry within which confirmatory investigations must have been performed (e.g. two years) may also help to prevent the entry of patients with inaccurate diagnoses.

Ensuring that only true IBD patients are recruited can be achieved by adhering to standard diagnostic criteria such as those proposed by Riis for Crohn’s disease, i.e. that two out the following diagnostic criteria should be present; typical history, histological or endoscopic confirmation, radiological evidence of Crohn’s disease and lastly, the presence of enterocutaneous fistula(e) or Crohn’s-related abscess(es) {Riis 1990}. A similar system has been proposed for confirmation of ulcerative colitis {Lennard-Jones 1989}. Using such criteria maintains patient homogeneity by excluding patients with rarer forms of colitis such as microscopic...
colitis, collagenous colitis and eosinophilic colitis, whose symptoms may be indistinguishable from those with ulcerative or Crohn's colitis.

3.1.2 Disease extent
Deciding on which disease phenotypes to include in an IBD trial will depend on the proposed role for the study drug. In addition, the study’s end-points will affect patient selection. For example, Crohn’s disease studies in which biopsy material or endoscopic appearances are used to assess efficacy will probably necessitate recruitment of patients whose colonic involvement or ileal disease is readily accessible by colonoscopy. Secondly, if a drug is thought to be useful in fistulising disease, it will be important to decide whether to include only patients with perianal enterocutaneous as opposed to other forms of fistulae (e.g. recto-vaginal), since score systems are generally designed for those with enterocutaneous lesions (see 3.3.1). Whether to include patients with ‘proctitis only’ is also an important decision, since these patients tend to experience fewer abdominal, systemic and extra-intestinal symptoms (Meucci, Vecchi, et al. 2000) and may respond better to topical than systemic treatments. This relates particularly to studies whose end-point is based on changes in global clinical activity indices, since these score methods may be weighted by the presence of systemic symptoms.

3.1.3 Disease activity for trial entry
Setting patients’ disease activity limits for trial entry will depend mostly on whether the study aims to examine the drug’s ability to induce or maintain remission. Consequently, a drug’s potential role in IBD treatment will be governed by its mode and predicted speed of onset of action. For example, drugs with anti-inflammatory mediator activity such as corticosteroids, 5-aminosalicylates (5-ASAs) and targeted anti-cytokine treatments are likely to produce more rapid symptomatic improvement than those which act predominantly by producing marrow suppression (e.g. azathioprine, mycophenolate mofetil).
3.1.3.1 Inducing remission of active disease

IBD activity may be defined by clinical activity indices and/or practical features, such as requirement for intravenous corticosteroids. It is important to decide whether to include the latter group, since patients whose disease is severe enough to require hospital admission are often clinically unstable and have increased likelihood of requiring urgent surgery. Ethically, it is difficult to include such patients in trials of therapies whose onset of action is greater than a few days and whose immunosuppressant action is not potent, since they are relatively unlikely to experience any benefit. Patients with severely active disease can be defined by using a CDAI score of greater than 400 or 450 points, whereas remission may be set as a score less than 150 points (see Section 3.3.1). Truelove and Witts’ criteria for severe ulcerative colitis may be used similarly (Section 3.3.2).

Patients’ IBD medication may also be used to determine a study’s target population. One such group, who may account for approximately 36% of Crohn’s patients, are those who are ‘steroid-dependent’, i.e. in whom withdrawal of oral corticosteroid provokes symptomatic deterioration and who therefore require a minimum maintenance dose. Alternatively, ‘steroid-resistance’ is a term used to identify a group of patients with moderate to severely active disease, which does not improve despite maximal doses of oral corticosteroids. Steroid-resistance has been found to account for up to 20% of Crohn’s patients in one prospective study {Munkholm, Langholz, et al. 1994}. Whichever treatment group is used to define a study population, it is important to establish the limits of dosage and duration of that treatment in the trial’s entry criteria. For example, failed withdrawal of oral corticosteroids on at least two occasions during the 12 months prior to study entry may define steroid-dependence, or steroid-resistance may be defined as the persistence of active disease despite at least 14 days of at least 40mg oral prednisolone daily.

3.1.3.2 Maintenance treatment trials

Similar methods to those described above can be used in maintenance trials. Symptomatic remission is usually required for trial entry, defined clinically by a low disease activity score, for a specified minimum time period e.g. six months. Given that retrospective calculation of disease activity scores is relatively
unreliable, using treatment limits is an additional means of defining remission, e.g. those without initiation or increased dose of corticosteroids within six months of study entry. Additionally in Crohn’s disease trials, surgical resection of affected bowel may be useful as the trial’s start-point, since it defines a group of patients whose chances of developing disease relapse are equivalent. Finally, a treatment’s efficacy at inducing and maintaining remission can be assessed within a single trial by incorporating the option of ‘open-labelled treatment’ at the end of the trial’s initial placebo-controlled follow-up period. For example, a single infusion of infliximab has been shown to induce remission in 39% patients with moderately active Crohn’s disease and to maintain remission in virtually all those in remission who went on to receive 8-weekly infusions for the following eight months {Rutgeerts, D’haens, et al. 1999}.

3.1.4 Exclusion criteria
3.1.4.1 Patient-related issues
In addition to groups commonly excluded from many clinical trials of new drugs, such as pregnant or breast-feeding women, children and patients who self-administer illegal substances, there are certain disease-related problems which should be considered when designing exclusion criteria for IBD trials. Firstly, it is important to establish prior to entering a patient with active disease into a trial that their symptoms are wholly accounted for by IBD. Specifically, infectious enteritis, an undetected colonic neoplasm and bile salt malabsorption following terminal ileal resection in Crohn’s patients should be excluded as possible causes of abdominal symptoms. Secondly, patients with Crohn’s-related strictures, whose symptoms are thought to be due more to irreversible fibrosis rather than treatable inflammation, should probably be excluded from trials of systemic treatments, since they are unlikely to gain much benefit from further immune modulation. Finally, patients with a permanent end-ileostomy or ileo-anal pouch are relatively unsuitable for clinical trials of systemic treatments for active Crohn’s disease, unless some adjustment is made to the clinical activity index being used to assess efficacy. Most Crohn’s disease activity indices are dependent on the number of loose bowel actions per day, which in these patients will almost always be high, giving a falsely high score.
Patients who are likely to be poorly compliant with treatment or with the frequency of a trial's follow-up visits should also be excluded. It is important to recognise that the number of such patients will be proportional to a protocol’s complexity. In particular, the ability to attend study visits of those in full-time employment, with dependent family members and those living a long distance away from the study centre should be considered when designing a trial, as these groups may have difficulty attending frequent appointments. Given that such patients may represent the majority of the target trial population, determining the frequency of follow-up is relevant to patient recruitment. This is an important consideration in IBD clinical trials, since recruitment rate is unlikely to exceed an average of 0.75 patients per month, depending on a study centre’s patient population (personal communication; Elan Pharmaceuticals Ltd.).

3.1.4.2 Drug-related issues

Some exclusion criteria will be idiosyncratic to a particular new drug because of previously known or predicted adverse events. In particular, any medication or disease known to produce an adverse event in combination with the study drug should be considered as exclusion criteria. Examples of such interactions might include patients on warfarin in a trial of subcutaneous heparin, patient taking allopurinol in a trial of azathioprine or 6-mercaptopurine and patients previously exposed to murine proteins in trials of mouse chimeric monoclonal antibodies. This problem is likely to affect recruitment to future IBD trials, since the chance that potentially eligible patients will have been exposed to murine proteins is likely to increase whilst infliximab is prescribed. Finally, patients taking treatments unlicensed for IBD, such as cyclosporin, mycophenolate mofetil and methotrexate, are often excluded from trials of other systemic immunotherapies. In addition to the potential risk of combining two ‘experimental’ treatments, the reason for this is largely legal, since it is difficult for a pharmaceutical company to obtain protective indemnity for adverse events which may be due to an unlicensed treatment.
3.2 Study design

3.2.1 Sample size
Estimation of the most appropriate sample size for a prospective study using power calculations is extremely important. Statistically, calculating the sample size is a means of maximising the chances of detecting a real and significant difference between two treatments. The smaller the predicted difference between treatment groups for comparison, the larger the trial must be to avoid wide confidence intervals and thus an inconclusive study result. Calculating sample size also has ethical and financial implications, since it is difficult to justify exposing large numbers of patients to a new treatment if the chances of detecting a treatment benefit are slight, unless the benefit itself is substantial.

3.2.2 Randomisation and choice of comparisons
Randomised double-blind placebo-controlled trials are the most appropriate means of assessing new therapies, although open studies can be useful for an initial assessment of a treatment’s potential efficacy. Randomisation can be performed using a concealed code system held ideally by a single investigator who remains separate from the trial until data analysis is complete. The treatment choice can be allocated numerically using a computer-generated random list for small trials. In larger multicentre trials however, treatment blocks can be used, the number within each randomly ordered block being dependent on the ratio of active to placebo treatments, to ensure that patients in each centre have an equal chance of being exposed to the study drug.

The main advantage of placebo-controlled trials, compared to trials of two active treatments, is that smaller numbers of patients are needed to show ideally larger differences between treatment and placebo groups. This affords placebo-controlled trials an additional ethical advantage, since less fewer patients overall are exposed to the new untested treatment. Often, another active drug or positive control is chosen for comparison, such as in the case of prednisolone versus budesonide for Crohn’s disease {Rutgeerts, Lofberg, et al. 1994}. One disadvantage of choosing a commonly used drug for comparison is the inevitable selection bias towards the ‘current treatment’, since patients entering the trial are likely to have demonstrated their tolerance of this treatment already. This problem
is difficult to avoid in trials comparing two active drugs, since it is impractical if not unethical to recruit only patients with newly diagnosed, previously untreated disease.

3.2.3 Stratifying groups
Stratification of patients prior to randomisation is useful in IBD trials in which patients with different disease phenotypes may be expected to have a differential response to the study drug. For instance, in a trial of a therapy for active disease, treatment groups can be randomised according to disease extent: ileal, colonic, ileal and colonic for Crohn’s disease or pancolitis versus left-sided disease in ulcerative colitis {Hanauer, Schwartz, et al. 1993}. Other factors which may predict response and therefore may be considered useful for stratification include smoking habit {Lindberg, Jarnerot, et al. 1992} and presence of active fistulising disease. Furthermore, in large multicentre studies, stratifying for the presence of rarer IBD phenomena, such as enterocutaneous fistulae, may aid generation of subgroup analyses of the effects of a study drug on these patients. Alternatively, patients can be stratified by concurrent medication, as a surrogate indicator of disease severity (e.g. prednisolone and 5-ASA, prednisolone, 5-ASA and azathioprine, and 5-ASA alone). When an optional investigation is included in a trial’s protocol, e.g. colonoscopy, it may be useful to stratify the groups for this test, particularly if the potential group size of such patients is likely to be too small to produce meaningful comparative data if left to chance alone. Finally in trials of a drug for use in maintaining remission, stratification by remission duration at study entry may be useful.

3.2.4 Blinding
Double-blinding is important in IBD trials, since the placebo effect is particularly strong, accounting for at least 10% of remissions and 30% of those who experience clinical benefit {Ilnyckyj, Shanahan, et al. 1997}. Blinding of the investigator is also important, so as to avoid bias in the objective clinical assessment of those who receive the therapy being studied. Retaining blinding of the investigator until the end of data analysis is relatively difficult however, particularly if there is an obvious early symptomatic benefit with the active drug. Furthermore, the trial drug may have systemic effects which are readily identified
by safety analyses. For example, IL-10 has been found to produce haemolysis in some patients, cyclosporin will cause renal impairment unless the plasma level is kept within a narrow therapeutic window and monoclonal antibody treatments may produce a transient rise in serum IgG. Where possible, results which may unblind the investigator can be shown instead to an independent safety monitor, who will disclose such results to investigators only if considered so far out of the normal range as to require clinical action. Difficulties also arise if an endoscopic score system is used as a trial end-point, since there may be a temptation to over-interpret macroscopic improvement in those who have experienced symptomatic benefit. One way of assessing such somewhat subjective scores is to appoint an independent, blinded investigator to assess video-recordings of all colonoscopies.

3.2.5 Trial end-points
Determination of a study's primary end-point requires selection of either a finite event or time period post-intervention at which efficacy is assessed by a predetermined score system. Typical finite events in IBD trials include disease remission or relapse, initiation of 'rescue' corticosteroid therapy, initiation of rectal treatments for colitis, surgical intervention or closure of fistulae. Survival analysis using the Log-Rank probability test may be used to analyse this type of discrete data, by testing for significant differences between groups in the time from study start to development of the event.

Many trials, however, choose a fixed time-point post-intervention at which to compare efficacy between groups. Remission of Crohn's disease is usually defined by a CDAI score of less than 150 points. Similarly, this arbitrary cut-off point may be used to define relapse in maintenance trials. A fall of 70 points in the CDAI has also been used in some trials to define a clinical response {Targan, Hanauer, et al. 1997}. This added measure may be useful in interpreting the results of a study of a treatment whose efficacy is marginally better than placebo, in that confidence intervals may be narrower than if remission data were used. However, given that many trials quote a standard deviation of the CDAI to be between 50 and 65 points in patients with mild to moderately active disease, the validity of this arbitrary measure of a response is questionable. Alternatively, the percentage change in CDAI at a given time-point compared to the baseline pre-
treatment score is frequently used as a measure of efficacy in Crohn’s disease trials. By contrast, the definition of remission is less distinct for ulcerative colitis trials, although Powell-Tuck et al. suggest that a score of zero points using their system implies remission {Powell-Tuck, Bown, et al. 1978}. Instead, ulcerative colitis trialists frequently analyse differences in the components of their score system at a fixed time-point. For example, a trial of balsalazide for maintenance of remission in ulcerative colitis used the cumulative frequency of patients’ nocturnal symptoms at 12 months as an end-point {Green, Lobo, et al. 1998}. Few trials choose endoscopic or histological remission as end-points for reasons described in Section 3.3.4.

Biochemical end-points, such as C-reactive protein (CRP) or serum albumin, measured at fixed time-points after study entry have the advantage of being a objective measure of disease activity. In some situations, such as distal colitis or proctitis, CRP or albumin may not be sensitive enough measures, however, since their relationship with disease activity is unlikely to be linear.

The percentage-change in corticosteroid dose may also be used as an end-point. These data are less easy to analyse in small pilot studies, however, since wide variations are likely to occur between patients. Additionally, such analyses necessitate patients taking corticosteroids from the outset of the study and fixing the dose at the study’s start. Such trials will, therefore, exclude patients who find the adverse effects of corticosteroids intolerable, leading to a reduction in the potential pool of patients available for recruitment.

Finally, adverse events may be selected as study end-points if a treatment has been specifically designed to have a low side-effect profile. For example, corticosteroid-related side-effects were chosen as end-points in trials of budesonide compared with prednisolone {Rutgeerts, Lofberg, et al. 1994}.

3.3 Data collection

The essential data collected in an IBD clinical trial is dependent largely on which clinical end-points are used to assess efficacy and which parameters are used to judge safety and tolerability. A common general criticism of IBD trials is their
dependence on subjective symptom reporting for determining efficacy. Although obtaining data from endoscopic assessment and/or biopsy samples seems a more objective measure of active disease, these techniques remain confounded to a degree by subjective observer bias and sampling error. IBD activity scores have been developed, therefore, as composite measures of symptom severity and objective evidence of inflammation.

3.3.1 Crohn’s disease activity scores
The Crohn’s disease activity index (CDAI) was originally developed for a large multicentre double-blind, placebo-controlled study of mesalazine in patients with active Crohn’s disease {Best, Becktel, et al. 1976}. Since its validation by this study, the CDAI is the most widely used clinical activity score in Crohn’s studies. It relies on patients scoring their symptoms daily for one week (general well-being, abdominal pain, number of loose bowel actions, presence of extra-intestinal manifestations of IBD, use of anti-diarrhoeal medication) and some objective measures of activity (presence of fever >38°C, weight loss as a percentage of expected body weight, haematocrit, presence of abdominal mass). Factors are weighted with multiplication factors and the end score is defined as active if greater than 150 points, scores of greater than 400 points defining patients with severely active disease.

Criticisms of the CDAI include its dependence on patients’ use of a 7-day diary card, its relative weighting of subjective terms, its lack of inclusion of any inflammatory markers (e.g. ESR or CRP) and its multiple calculations, making it both impractical and prone to error {Hodgson & Mazlam 1991}. Consequently, others have suggested simpler schemes such as the Harvey-Bradshaw index, which relies on a single day’s data and correlates well with the CDAI (r=0.9) {Harvey & Bradshaw 1980}. Van Hees also suggested a score system which is more dependent on objective measures of inflammation such as ESR, CRP, platelet count, albumin {van Hees, Van Elteren, et al. 1980}. Although the Van Hees index is less prone to inter-observer error, it correlates weakly with more subjective-based scores such as the CDAI, suggesting that it may define a different type of active IBD patient {Myren, Bouchier, et al. 1984}. Finally, a validated score system exists for fistulising disease {Irvine 1995}, which focuses
on perianal disease and has been used in recent trials of infliximab {Present, Rutgeerts, et al. 1999}.

3.3.2 Ulcerative colitis activity scores
In contrast to Crohn’s disease, opinion remains divided as to the most useful and reproducible score system for measuring clinical activity in ulcerative colitis trials. Truelove and Witts’ criteria for mild, moderate and severe disease are widely used for defining disease activity for trial entry {Truelove & Witts 1955}, but are not sensitive enough to identify small changes in disease activity at follow-up visits {Singleton 1987}. In addition, a slightly modified Truelove and Witts’ score system correlates poorly with endoscopic and biopsy assessment of disease {Gomes, du, et al. 1986}. The Powell-Tuck score has been validated in clinical trials as a more sensitive measure of disease fluctuation, with the advantage of including sigmoidoscopic appearance as one of its parameters {Powell-Tuck, Day, et al. 1982}. Rachmilewitz developed a similar score system to Powell-Tuck with the addition of the investigator’s global assessment of patient well-being {Rachmilewitz 1989}. The lack of consensus on the most useful score system for ulcerative colitis trials has led many investigators to develop their own indices, with the disadvantage that it remains difficult to compare the results of different trials with one another.

3.3.3 Quality of life measures in IBD
The value of collecting data on quality of life measures has been established largely by results of cancer therapy trials. The techniques used for measuring quality of life have now been applied to most medical conditions including IBD. Many have argued that the way in which IBD impacts on lifestyle varies widely between patients and that quality of life is therefore a more reliable measure of health status than activity indices alone {Garrett & Drossman 1990}. The McMaster IBD quality of life index is now one of the most commonly used indices in IBD studies {Guyatt, Mitchell, et al. 1989}, although other investigators have suggested that lifestyle problems are disease-specific and have therefore modified the index for separate use in ulcerative colitis and Crohn’s disease patients. Increasingly, quality of life scores are included in IBD clinical trials and provide useful supplementary information to clinical activity indices, since they
are affected by a drug's adverse events. This is highlighted in studies of budesonide compared to prednisolone, which showed that although budesonide was associated with a lower frequency of adverse events, there was no significant difference in quality of life score between treatment groups {Bar-Meir, Chowers, et al. 1998}.

3.3.4 Endoscopic score systems

The role of colonoscopic assessment of disease activity as an outcome variable in IBD trials remains controversial. Firstly, there are many reports which suggest that mucosal appearance at colonoscopy correlates poorly with symptoms, particularly in Crohn's disease {Landi, Anh, et al. 1992}, in which mucosal activity frequently persists despite clear symptomatic remission {Modigliani 1990 52 /id}. Secondly, patients may be more reluctant to take part in a study if the protocol includes mandatory colonoscopic examination, although a study of mesalazine for Crohn's maintenance found that 68% did not object to undergoing annual colonoscopy for the purpose of the trial alone {Kennedy, Blair, et al. 1998}. A widely used endoscopic score system for colonoscopic evaluation of Crohn's disease has been designed by Mary and Modigliani {Mary & Modigliani 1989}, which has been used recently in multicentre studies of infliximab in active Crohn's disease. This score system divides the examination into five segments for comment; terminal ileum, ascending colon, transverse colon, descending colon and rectum. The total score is based on cumulative segmental scores for the percentage of the mucosa affected by ulceration and the presence of other inflammatory features e.g. pseudo-polyps. The system designers advise that a video describing the scoring method is shown to all colonoscopists before starting a study and that videos of each procedure are stored for scoring by an independent investigator.

The value of colonoscopic assessment is less disputed for ulcerative colitis, although as yet, no standard score system for total colonoscopy has been designed. In addition, colonoscopic assessment is inappropriate for studies involving patients with severe colitis requiring in-patient admission, in whom the risks of colonic perforation are relatively high. Most score systems are derived from rigid sigmoidoscopic evaluation, the most robust of which is that of Baron et al.
{Baron, Connell, et al. 1964}. This system uses four terms to describe the rectal mucosa; normal (0), loss of vascular pattern only (1), contact bleeding (2) and spontaneous haemorrhage +/- mucopus (3). The system has also been adapted as part of the Powell-Tuck score, where 0 does not score, 1 and 2 are equivalent to one point and 3 is two points.

3.3.5 Evaluation of histology in IBD trials

Using histological samples in IBD trials is not recommended as a primary end-point due to sampling error incurred by trying to collect sequential samples from the same site as that taken pre-treatment. This is particularly applicable to Crohn’s disease, given its patchy mucosal involvement. Furthermore, scoring of inflammation in biopsy material is prone to bias and it is therefore essential that the histologist is blinded. Nevertheless, biopsy samples are helpful for qualitative assessment of the effect of a treatment on IBD pathogenesis and may contribute to the understanding of disease mechanisms {Yacyshyn, Bowen-Yacyshyn, et al. 1998}. Few standardised score systems exist and most are constructed by trialists themselves. However, a classification system has been described by Saverymuttu et al. for Crohn’s disease biopsies {Saverymuttu, Camillieri, et al. 1986} and a similar system has been used recently in a trial of rectal budesonide {Danielsson, Lofberg, et al. 1992}.

3.3.6 Safety data and adverse events

Careful design of the adverse event section of a study’s case report form prevents the eventual recording of adverse events from becoming a lengthy and unwieldy process. The importance of attention to detail in recording safety data has been highlighted by the initial trials of infliximab for Crohn’s disease, in which a higher incidence of serum-sickness type reactions was identified in a patient subgroup who received a liquid formulation of the drug {Hanauer 1999}.

3.3.6.1 Treatment-specific events

In addition to the standard blood tests of renal and hepatic function, blood glucose, full blood count, inflammatory markers and urinalysis, some added safety analyses may be necessary, depending on knowledge of the treatment’s potential side effects from phase I and/or other clinical studies. For example, more detailed
monitoring of renal function have been incorporated in studies of cyclosporin, since it is known to be nephrotoxic. Similarly, trials of targeted murine chimeric monoclonal antibodies are likely to include monitoring of immune complex development and auto-antibody formation in the light of the findings described above.

3.3.6.2 Disease-related events

Recording of symptoms due to underlying IBD as adverse events becomes time-consuming if the effects of the study treatment are short-lived compared to placebo or other active treatment. It may be helpful in this context to standardise the reporting of adverse events which, in the investigator’s opinion, are most likely to be due to underlying disease. For example, rather than permitting investigators to record IBD-related abdominal pain as freetext, it may be useful to document this as ‘abdominal pain’ either as related or unrelated to IBD. Similarly, in order to establish as much uniformity of adverse event reporting between centres as possible, thresholds of severity of symptoms related to underlying IBD should probably be decided from the outset of the study. For example, investigators may decide that only abdominal symptoms which are worse than those at baseline merit reporting.

Should interventions or changes in the patient’s state constitute withdrawal from a study, strict criteria also need to be established to ensure uniform reporting. If the safety profile of a new treatment is unclear, patients who are withdrawn because of relapse requiring surgery should probably remain at least in the trial’s safety analysis. Long-term follow-up of safety data is more difficult, but clearly important for treatments designed specifically for use in a chronic condition like IBD. Recording of emergent malignancies has been highlighted in the context of anti-cytokine therapies and powerful immunosuppressant agents {Bickston, Lichtenstein, et al. 1999}.

Multicentre, double-blind, placebo-controlled trials will sometimes include an open-label treatment option for patients who initially have no response to treatment. This measure probably improves recruitment since patients realise that they will eventually have the option of receiving the new treatment, and also
maximises the number of patients exposed to the treatment. However, if the long-term effects of a new treatment are unknown, it may be better to consider withholding this open-label option to safeguard the placebo group, who may form an important group for comparison in the long-term, particularly if long-term adverse events develop.

3.4 Concomitant medication

3.4.1 Pre-trial medication

Some clinical trials of new IBD treatments demand that all other concomitant IBD drugs be stopped prior to study entry. Increasingly, physicians recognise that this is impractical and unrepresentative of clinical practice, since combination therapies for IBD are common. In addition, stopping existing medication presents ethical difficulties, particularly in placebo-controlled trials for active disease, since many patients’ symptoms may deteriorate following treatment withdrawal. A solution is probably to recommend that concomitant IBD treatments be kept at a stable dose for a specified duration prior to the study start, the time being dependent on the predicted remission-inducing properties of the drug. For example, it may be reasonable to suggest that patients taking corticosteroids or 5-ASA drugs remain on a constant dose for at least two or six weeks, respectively, prior to study entry, based on the evidence that improvements due to initiation or increased dose of these drugs would be expected within this time. Azathioprine has a longer onset of action than these drugs and improvements attributable to it have been documented as much as three to six months after its initiation {Present, Korelitz, et al. 1980}. This factor should be accounted for in study entry criteria.

3.4.2 Management of existing medication during trials

As discussed, concomitant IBD treatments should probably be kept at a constant dose during a trial, at least until the primary end-point is reached. This may not be practical for high dose corticosteroids, for example, for which a structured dose reduction schedule may need to be specified in the study’s protocol {Rutgeerts, Lofberg, et al. 1994}. Alternatively, the reduction rate may be left to the discretion of the physician and used subsequently to form a survival analysis between treatment groups.
3.4.3 Rectal treatments
These present difficulties in IBD trials of systemic treatments in which the selected clinical activity index depends on the appearance of the rectal mucosa. If such a score system is to be used as a study end-point in a trial for active disease, it may be wise to consider withdrawing rectal treatments at least two weeks prior to study entry, to allow a ‘wash-out’ period prior to commencing the study drug. Again this may present ethical difficulties for those with severely active distal colitis randomised to placebo, for whom an alternative is to keep the dose of rectal treatments constant from a defined point prior to study entry (e.g. two weeks) until after the study’s primary end-point. Flexible, as opposed to rigid, sigmoidoscopic assessment of the rectal mucosa may also be useful in this respect.

3.4.4 Rescue medication
The end-point of some trials is defined by the percentage of treatment failures i.e. who require ‘rescue treatment’, usually corticosteroids. If this is the case, precise details of rescue medications need to be outlined in the study protocol. For example, rescue treatment may be defined as the initiation or increase in dose of any therapy known to reduce disease activity. In addition to corticosteroids, 5-ASA drugs, and immunosuppressants 6-MP/azathioprine, methotrexate and cyclosporin should all be considered as rescue treatments. The antibiotics ciprofloxacin and metronidazole should also be included, since they have been shown to have disease-modifying effects, particularly in perianal Crohn’s disease. Finally, the introduction of an elemental or polymeric diet should be classed as a rescue treatment, particularly if paediatric patients are to be included in a study.

3.5 Data-handling
Analysis of data should use the intent-to-treat study population as opposed to the per protocol group. That is, analysis should include all patients who were randomised and who received the allocated treatment, including those who were withdrawn at any time after having received the study treatment. The principle of ‘last observation carried forward’ or ‘last/best value extended’ is usefully applied to data of patients who have received rescue medication or any other intervention such as surgery, corticosteroids or a blood transfusion, which might alter their disease activity score, or who have discontinued follow-up. With this method, the
last value prior to the intervention is ‘carried forward’ and used for all statistical analyses at subsequent time-points. An alternative approach is to withdraw such patients, although this results in progressively smaller cohorts of patients from which to make between-group comparisons, usually resulting in falsely high estimation of percentage of patients in remission, for example.

### 3.5.1 Missing data

Despite making every effort to minimise missing data, it will inevitably occur and methods of handling it in analyses are best established in a trial’s protocol. Missing follow-up visits or results of investigations which form part of a study’s end-point (e.g. sigmoidoscopic score or haematocrit) can be handled by applying the last observation carried forward principle for the missing data-point. Alternatively, if continuous data (e.g. CDAI, CRP) are missing at baseline, the mean of all patients at baseline may be used instead. For such patients, the imputed baseline value is then used for statistical comparisons with all subsequent post-baseline visits (Singleton, Hanauer, et al. 1993). It has also been suggested that the baseline value should be substituted for a single missing value, although this method may be over-cautious compared to using LOCF data, particularly when considering visits many months after the study start. Use of the internet and digital telephone signals as means of data collection may reduce the likelihood of missing data.

### 3.5.2 Missing CDAI data

Opinions vary as to how best to handle missing diary card data for activity indices which require documentation of several days’ symptoms. Using continuous diary cards, contacting patients to remind them to fill in their cards at least seven days before important visits and arranging follow-up visits on a fixed day of the week may help to minimise this problem. One method of dealing with a CDAI variable whose diary card data are incomplete (e.g. stool frequency recorded for three days only) is to use the LOCF data for the variable alone (Winship, Summers, et al. 1979). Alternatively, the mean of the available days’ data can be calculated and imputed as the value for the missing days. Using this latter method, trialists may decide to define the maximum number of days of incomplete data permitted (e.g.
up to three days), more than which will invalidate the entire CDAI and necessitate use of the LOCF CDAI for that visit.
Chapter 4
A randomised placebo-controlled trial of natalizumab, a humanised monoclonal antibody to alpha-4 integrin in active Crohn’s disease.

4.1 Abstract

Background & Aims

a4 integrins are important mediators of leucocyte migration across vascular endothelium. This pilot placebo-controlled study aimed to assess the safety and efficacy of natalizumab (Antegren™), a recombinant humanised monoclonal antibody to a4 integrin, in patients with mild to moderately active Crohn’s disease.

Methods

Thirty patients with active Crohn’s disease (Crohn’s disease activity index [CDAI] ≥151 and ≤450) received a 3mg/kg infusion of natalizumab (n=18), or placebo (n=12) by double-blind randomisation. The study’s primary end-point was the change in CDAI at two weeks. Secondary end-points included the percentage of patients in remission (CDAI<150), or who required rescue corticosteroids at two weeks.

Results

At two weeks, the CDAI fell significantly from baseline post-natalizumab (mean 45 points) but not placebo (mean 11 points). Thirty-nine percent (n=7) of natalizumab-treated patients achieved remission at two weeks, compared with 8% (n=1) treated with placebo. By contrast, 33% (n=4) of placebo patients required rescue medication by two weeks compared with 11% (n=2) of natalizumab patients. A significant increase in circulating B and T cells was detected post-natalizumab only. The frequency of commonly reported adverse events did not differ significantly between groups.

Conclusions

A single 3mg/kg infusion of natalizumab was well-tolerated by Crohn’s disease patients, although the dose used may have been sub-optimal. The elevation in circulating lymphocytes post-natalizumab suggests that lymphocyte trafficking was affected.
4.2 Introduction
Integrins are heterodimeric glycoproteins which are widely expressed on leucocytes and are thought to be important mediators of leucocyte adhesion to vascular endothelium {Springer 1994}. The α4 integrin is expressed at a moderate or high level on almost all lymphocytes and to a lesser extent on monocytes and eosinophils {Hemler, Huang, et al. 1987}.

Clinical studies suggest that the interaction between α4β7 and MAdCAM-1 is particularly important in mediating leucocyte homing to gut mucosa {Farstad, Halstensen, et al. 1997}. Furthermore, studies of IBD patients have demonstrated that endothelial cells extracted from inflamed intestinal mucosa display increased α4-dependent adhesiveness to leucocytes in vitro {Binion, West, et al. 1998}.

The hypothesis that α4 integrin blockade could be useful therapy for IBD stems mainly from studies showing resolution of spontaneous colitis in captive cotton-topped tamarins by monoclonal antibody blockade of α4 integrins {Podolsky, Lobb, et al. 1993}. Clinical trials of such antibodies in Crohn’s disease have not been performed to date, although a pilot study of a humanised antibody to α4β7 integrin has demonstrated promising results in patients with ulcerative colitis {Feagan, McDonald, et al. 2000}.

Natalizumab (Antegren™) is a recombinant humanised antibody to human α4 integrin. Preclinical and Phase I studies have been described in Chapter 2.4. The aim of this study was to assess the safety and efficacy of natalizumab in patients with mild to moderately active Crohn’s disease in a randomised double-blind placebo-controlled trial.

4.3 Methods
4.3.1 Patients
Thirty-five patients with mild to moderately active Crohn’s disease, defined by a CDAI score ≥151 and ≤450 {Best, Becktel, et al. 1976}, were assessed for trial eligibility at an outpatient visit at least one week prior to the planned treatment date. Patients were recruited from the Centre for Gastroenterology, Royal Free
Hospital, London and from the Department of Gastroenterology, Royal Gwent Hospital, Newport.

Patients included in the trial were at least 18-years old and Crohn’s disease was required to have been confirmed by two or more of the following diagnostic criteria at least three months prior to study entry: history, radiological or endoscopic intestinal appearance, histology, the presence of Crohn’s-related fistula(e) or abscess formation {Riis 1990}. Female participants were required to have a negative pregnancy test at study entry and to use effective contraception throughout the study follow-up period. Patients receiving oral corticosteroids (≤40mg prednisolone or ≤9mg budesonide daily) were included, provided that the dose had not been altered within two weeks of study entry. Likewise, patients receiving 5-aminosalicylic acid-derived drugs or azathioprine / 6-mercaptopurine were eligible, provided that treatment had not been initiated or the dose increased within two and four months of study entry, respectively. The local ethics committees of both participating hospitals approved the trial and written informed consent was obtained from all patients prior to study entry.

4.3.2 Exclusion criteria
The following patients were excluded from the study: patients whose CDAI score exceeded 450 or who were inpatients on account of Crohn’s disease, patients receiving cyclosporin, methotrexate or tacrolimus therapy, patients who had undergone intestinal surgery up to three months before study entry or who were thought likely to require surgery within three months of study entry, patients with an ileostomy or colostomy, patients with laboratory-confirmed intestinal infection and patients known to have had a malignant neoplasm at any site. Patients weighing more than 100kg and pregnant or breast-feeding women were also excluded.

4.3.3 Design
A sample size of 12 patients was necessary to detect a difference of 103 points between pre-treatment and week two mean activity index values with 80% power, assuming a standard deviation of differences of 155, using a paired t-test with 5% two-sided significance level. The difference of 103 points was based on the
findings of a previous randomised placebo-controlled trial of infliximab, in which the week two CDAI was 212 points (SD +/-90), compared with 312 +/-55 points at baseline in those who received infliximab {Targan, Hanauer, et al. 1997}. The number of natalizumab-treated patients was increased to 20 to allow for early withdrawals and provide sufficient preliminary safety data. It was our intention that patients should be assigned treatment with natalizumab or placebo in a two-to-one randomisation, i.e., 20 natalizumab to 10 placebo. However, a randomisation error at one centre (RFH) resulted in the size of the eventual active treatment and placebo groups being 18 and 12 patients, respectively (Figure 4.1). Individual randomisation concealment codes were held by the trial’s sponsor and each hospital’s pharmacy for emergency use, none of which were opened during the study. Investigators and patients remained blinded to the randomisation codes until data analysis was complete.

Patients received a single 3mg/kg intravenous infusion of natalizumab (Antegren™, Elan Pharma Ltd., Letchworth, U.K.) or placebo over 30 to 75 minutes. Natalizumab (5mg/ml) was formulated in a solution of 10mM phosphate-buffered 0.02% polysorbate 80 adjusted to pH 6 with hydrochloric acid and was diluted to 100ml in 0.9% saline for administration. Placebo consisted of 10mM phosphate-buffered 0.02% polysorbate 80 similarly diluted to 100ml in 0.9% saline. Patients were kept under direct observation for a minimum of six hours from the start of the infusion.

Patients were reviewed at one, two, four, eight and 12 weeks following treatment. Patients kept a daily symptom diary card for at least seven consecutive days prior to each outpatient visit which, together with examination and haematological findings, was used to calculate their CDAI score (see Appendix I). Remission was defined as a CDAI score of less than 150 points. ‘Rescue’ medication was defined as the initiation or increased daily dose of any of the following drugs: corticosteroids (intravenous or oral), 5-aminosalicylates or azathioprine / 6-mercaptopurine. Quality of life was assessed by the IBD questionnaire (IBDQ) {Guyatt, Mitchell, et al. 1989} completed prior to and four weeks after treatment. Venous blood was taken at each visit for analysis of the following: C-reactive protein (CRP), erythrocyte sedimentation (ESR), full blood count, serum
biochemical screen (including pregnancy test in females), serum natalizumab and anti-natalizumab antibody concentrations. The latter were measured at Athena Neurosciences, South San Francisco, USA, by a previously validated ELISA method (Holsztynska, Kung, et al. 1995). The proportion of peripheral blood T cells (TCRαβ+) and B cells (CD19+) were also measured at all follow-up visits using fluorescence-activated cell-sorter (FACS) analysis (Becton Dickinson, Oxford, UK) as described previously (Amlot, Tahami, et al. 1996).

4.3.4 Statistical analysis
The change in CDAI at two weeks post-infusion in the intention-to-treat population was used as the study end-point and was analysed using a two-tailed paired t-test (significant p value <0.05). Secondary end-points included the percentage of patients in remission (CDAI<150), and the percentage who required rescue medication at two weeks post-infusion. In order to try to account for improvements in CDAI score due to rescue medication, the last CDAI value recorded prior to commencing rescue medication was carried forward for statistical analyses at subsequent time-points (i.e. last observation carried forward or LOCF data). LOCF data were also used for patients who were withdrawn from the study.

Paired and unpaired t tests were used for within-group and between-group analyses of parametric data, respectively. Wilcoxon signed rank and Mann-Whitney tests were used for equivalent analyses of non-parametric data. Chi-squared test was used to test for differences in remission rates between groups. Spearman correlation tests were used to examine the relationship between response to natalizumab, defined by the change in CDAI at week two, and the following: CDAI pre-infusion, change in CRP at week two, age and disease duration.

4.4 Results

4.4.1 Demography
The demographic characteristics of the 30 patients who received natalizumab or placebo are shown in Table 4.1. Five patients were screened but not included in the study due to blood test abnormalities or personal choice. The groups were
well-matched for age, sex, height, ethnicity, disease duration and IBDQ score, although mean pre-treatment weight was greater in the natalizumab group (p=0.02).

The mean pre-treatment CDAI of the natalizumab group (258 points) was less than that of placebo (273), but the difference is not significant (p=0.95). One natalizumab group patient had a pre-treatment CDAI of 122 points which was accounted for by a combination of an unexpected change in haematocrit and calculation error in one sub-section of the CDAI. This was not recognized until after treatment and the patient’s data were therefore included in all analyses. At study entry, the mean prednisolone dose (12mg natalizumab group; 15mg placebo group) and the proportions of patients receiving 5-aminosalicylates or azathioprine / 6-mercaptopurine did not differ significantly between groups. The flow of patients through the trial is shown in Figure 4.1.

Table 4.1  Demographic characteristics of patients at study-entry

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo</th>
<th>Natalizumab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=12</td>
<td>n=18</td>
</tr>
<tr>
<td>Mean disease duration/ years (SD)</td>
<td>8.4 (6.0)</td>
<td>8.5 (9.6)</td>
</tr>
<tr>
<td>Mean age/ years (SD)</td>
<td>34.4 (8.8)</td>
<td>36.0 (13.2)</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>5/7</td>
<td>7/11</td>
</tr>
<tr>
<td>Mean weight/ kg (SD)</td>
<td>57.5 (8.6)</td>
<td>66.4 (10.6)</td>
</tr>
<tr>
<td>Mean height/ m (SD)</td>
<td>161.5 (7.1)</td>
<td>166.5 (8.7)</td>
</tr>
<tr>
<td>Ethnicity Caucasian/ Asian</td>
<td>11/1</td>
<td>17/1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease site</th>
<th>Placebo</th>
<th>Natalizumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal or ileo-caecal alone</td>
<td>5 (42)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Colonic alone</td>
<td>3 (25)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Ileal and colonic</td>
<td>4 (33)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Perianal</td>
<td>4 (33)</td>
<td>5 (28)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Activity</th>
<th>Placebo</th>
<th>Natalizumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean CDAI (range)</td>
<td>273 (191-420)</td>
<td>258 (122-436)</td>
</tr>
<tr>
<td>Mean IBD quality of life score (range)</td>
<td>118 (78-144)</td>
<td>121 (74-167)</td>
</tr>
<tr>
<td>Mean CRP/ mg/l (SD)</td>
<td>35 (42)</td>
<td>14 (12)</td>
</tr>
<tr>
<td>Mean serum albumin/ g/l (SD)</td>
<td>42 (4)</td>
<td>41 (3)</td>
</tr>
<tr>
<td>Mean ESR/ mm/hr (SD)</td>
<td>27.5 (15.9)</td>
<td>26.4 (13.4)</td>
</tr>
</tbody>
</table>

| Medication at week 0            | Placebo          | Natalizumab       |
|                                | n=12             | n=18              |
| Nil                            | 1 (8)            | 3 (17)            |
| Prednisolone/ budesonide       | 9 (75)           | 10 (56)           |
| Azathioprine                   | 2 (17)           | 6 (33)            |
| 5-amino salicylic acid         | 9 (75)           | 13 (72)           |
| 5-amino-salicylic acid alone   | 2 (17)           | 3 (17)            |
4.4.2 Clinical response to treatment
At two weeks, natalizumab-treated patients achieved a statistically significant mean reduction in CDAI of 45 points (p=0.02), i.e. from 258 (range 122-436) to 213 points (range 46-413), although this is not significantly different from the mean change of eleven points which occurred in placebo patients (p=0.2, Figure 4.2). At four weeks, the reduction in CDAI in natalizumab-treated patients remained significant (p=0.01), but note that by this time many patients had commenced rescue therapy (9/18 [50%] natalizumab group; 8/12 [67%] placebo group), hence LOCF data were used for these patients. In placebo patients, the change in mean CDAI from baseline values at weeks 2 and 4 was not statistically significant.
By two weeks, 39% (n=7) of the 18 patients who received natalizumab had experienced remission, compared with 8% (n=1) of the placebo group (Figure 4.3; observed differences between groups not statistically significant). Remission was sustained to at least week 12 in two of the natalizumab-treated patients, the remainder having required rescue therapy at a median of 22 days post-infusion (range 17-89 days). Overall, by two weeks, ‘rescue’ treatment had been commenced in 33% (n=4) of placebo patients compared with 11% (n=2) of those who received natalizumab (Figure 4.3). Finally, 50% (n=9) of natalizumab-treated patients and 59% (n=7) of placebo-treated patients continued to have active Crohn’s disease at two weeks, but had not commenced rescue therapy. Disease duration was the only clinical factor which correlated significantly with response to natalizumab (r=-0.54; p=0.02).
Clinical status of Crohn’s disease patients two weeks after single infusion of natalizumab 3mg/kg or placebo.

Figure 4.3 Observed differences between groups are not significant.

4.4.3 Quality of life
A significant improvement in mean IBDQ score occurred from 121 points at baseline to 140 points at four weeks in patients who received natalizumab (p=0.004). No significant improvement in IBDQ score was observed in placebo-treated patients.

4.4.4 Inflammatory markers
Patients who received natalizumab experienced a significant reduction in CRP at two and four weeks compared to baseline (p=0.02 at both time-points), and a significant reduction in ESR at four weeks only (p=0.04). These changes did not differ significantly from those which occurred in the placebo group. In placebo patients however, the changes in CRP and ESR at two and four weeks were not significant compared to baseline. Platelet counts did not change significantly in either treatment group between baseline and four weeks. Finally, there were no significant differences between groups in any of these inflammatory parameters at any point in the study.
4.4.5 Pharmacokinetics and antibody formation

The mean plasma half-life of natalizumab was 4.8 days (Figure 4.4). The mean maximum serum concentration (Cmax) achieved at one hour post-infusion was 52.8 mcg/ml and most patients had detectable serum levels of natalizumab at four weeks, the mean serum concentration being 0.99 mcg/ml. Two patients (11%) developed transient low-titre non-anti-idiotypic antibodies to natalizumab, detectable at a single visit only within the 12-week trial follow-up period. One of these patients experienced symptomatic remission from week one to at least week 12, having developed detectable anti-natalizumab antibodies at week eight. Antibodies to natalizumab were detected at week four in the other patient, who showed no clinical response to natalizumab.

Figure 4.4 Mean serum natalizumab concentrations of 18 Crohn’s disease patients following a single 3mg/kg infusion.

4.4.6 Tolerability and adverse events

The infusion was generally very well-tolerated. The most common adverse events, reported in at least 20% patients during the 12-week follow-up period, were headache (50% of each treatment group), ‘Crohn’s disease’ (natalizumab 39%;
placebo 42%) and abdominal pain (22% natalizumab; placebo 17%). The frequency of these events did not differ significantly between groups.

Six natalizumab-treated patients required admission on account of problems related to Crohn's disease, namely worsening of symptoms in five patients and chronic anaemia requiring blood transfusion in one patient. Two of the patients admitted for symptomatic relapse had initially achieved remission at two weeks post-infusion, one patient had experienced a CDAI reduction of 70 points at one week and the remaining two patients had not responded to natalizumab. Overall, the median time from natalizumab-infusion to admission was 48 days and ranged from 16 to 66 days.

Two natalizumab-treated patients (included in admission group above) and one placebo-treated patient required surgical resection of their Crohn's disease at 66, 69 and 70 days, respectively. Two of these patients (one natalizumab, one placebo) were withdrawn from the study and a further placebo-treated patient was withdrawn having failed to attend follow-up visits beyond four weeks post-infusion. All three withdrawals occurred at least 50 days after the study's start, hence these patients' data were included in the primary end-point analyses at two-weeks.

4.4.7 Immunological parameters

A significant increase in mean circulating lymphocyte counts occurred at one, two and four weeks post-infusion in natalizumab-treated patients only (Figure 4.5: $p<0.01$ each time-point compared to baseline). FACS analysis demonstrated that this increase comprised of both B (CD19+) and T cell (TCRαβ+) subsets (Figure 4.6). Change in CDAI at week 2 correlated negatively with disease duration ($R=-0.54$, $p=0.02$) and did not correlate with week 0 CDAI, age, CRP or lymphocyte count at week 2.
Figure 4.5  Mean lymphocyte counts post-natalizumab or placebo.

** Figure 4.5  ** p<0.01 compared to baseline values. Bars are SD.

Figure 4.6  Changes in B and T cells in natalizumab-treated patients.

** Figure 4.6  ** p<0.0001 and * p<0.01 compared to baseline
4.5 Discussion

This study shows that treatment with a single 3mg/kg infusion of natalizumab is well-tolerated by patients with active Crohn’s disease. Remission occurred at two weeks post-infusion in a greater proportion of natalizumab-treated patients than those who received placebo, but the difference between groups was not significant. Natalizumab patients achieved significant reductions in CDAI compared to baseline at two and four weeks post-treatment, although between-group comparisons with placebo patients did not reach statistical significance. However, CDAI reductions in natalizumab-treated patients were accompanied by improvements in CRP, ESR and IBDQ quality of life scores.

The effects of natalizumab at a dose of 3mg/kg appear to be relatively short-lived in patients with active Crohn’s disease. This is suggested by the finding that five of the seven patients who initially achieved remission at two weeks subsequently relapsed and required rescue therapy at a median of 22 days post-infusion. This may be related to the finding that the half-life of natalizumab of 4.8 days in IBD patients is shorter than 8.7 days observed in healthy volunteers {Athena 1996}. Additionally, in vitro leucocyte saturation studies now suggest that a minimum serum concentration of approximately 5μg/ml of natalizumab is required to produce appropriate saturation of at least 80% of membrane-bound α4 integrins (Elan Pharma Ltd.; unpublished data). The mean serum concentrations of natalizumab at two and four weeks post-infusion were 4.91mcg/ml and 0.99mcg/ml, respectively, suggesting perhaps that blockade of α4 integrins was suboptimal after a single 3mg/kg dose and that higher and/or more frequent doses might result in improved efficacy.

It is unclear why the half-life of natalizumab was shorter in Crohn’s disease patients than healthy volunteers. Patients in our study may have had a higher proportion of circulating α4+ cells than healthy volunteers, although previous studies of peripheral blood α4+ T cells suggest that this is unlikely {Meenan, Spaans, et al. 1997}. Interestingly, the serum half-life of a recombinant humanised anti-TNFα antibody (CDP571) was also found to be shorter in Crohn’s patients than that predicted by a phase I study {Stack, Mann, et al. 1997}. This may be related to the higher levels of tissue and/or circulating TNFα concentrations found
in IBD patients compared with unaffected individuals {Breese, Michie, et al. 1994}.

All serious adverse events recorded during the study were due to worsening of underlying Crohn's disease as a consequence of lack of sustained efficacy, and were therefore thought to be unrelated to natalizumab itself. The low-titre antibodies formed by two patients (11%) were not thought to be clinically significant, since they were not anti-idiotypic. A similar proportion of multiple sclerosis patients developed transient low-titre antibodies after receiving two 3mg/kg infusions of natalizumab, one month apart, in a multicentre study {Tubridy, Behan, et al. 1999}.

A rise in both B and T cells accounts for the transient but significant lymphocytosis which was detected in natalizumab-treated patients alone. This effect appears to be consistent post-infusion of monoclonal antibodies to certain adhesion molecules in man and animal models. For example, lymphocytosis was seen following infusion of monoclonal antibodies to either \( \alpha 4 \) alone {Podolsky, Lobb, et al. 1993} or to \( \alpha 4 \beta 7 \) integrin {Hesterberg, Winsor-Hines, et al. 1996} in the cotton-topped tamarin. Furthermore, a clinical trial of monoclonal antibody to ICAM-1 also produced transient elevation of lymphocyte counts in patients with rheumatoid arthritis {Kavanaugh, Davis, et al. 1994}. A possible explanation for this finding in our study is that natalizumab selectively inhibits \( \alpha 4 \) integrin-mediated lymphocyte migration through vascular endothelium. Thus, \( \alpha 4^+ \) lymphocytes are forced to remain in the intravascular compartment and lymphocyte trafficking is interrupted. Although inflammation in IBD is thought to be mediated predominantly by T cells, the transient rise in B cells is likely to be related to the finding that \( \alpha 4 \) integrins are highly expressed on these cells {Andrew, Rott, et al. 1996}.

In conclusion, treatment of mild to moderately active Crohn's disease with blockade of \( \alpha 4 \) integrin by natalizumab (Antegren\textsuperscript{TM}) did not demonstrate significant efficacy compared to placebo in this pilot study. However, the single dose of 3mg/kg was probably suboptimal since it produced only short-lived reduction in symptoms, effects on circulating lymphocytes and inflammatory
mediators. The effects on lymphocytes are consistent with similar animal studies and support the hypothesis that lymphocyte trafficking to the human gut is mediated by $\alpha 4$ integrins. Further work is needed to establish the optimal dose level and dosing regimen required to assess the potential therapeutic benefit of natalizumab in patients with active Crohn’s disease.
Chapter 5
A pilot study of treatment of active ulcerative colitis with natalizumab, a humanised monoclonal antibody to alpha-4 integrin.

5.1 Abstract

Background and aims α4 integrins are important mediators of leucocyte migration across vascular endothelium. This open study aimed to assess the safety and efficacy of natalizumab (Antegren™), a humanised antibody to α4 integrin, in patients with active ulcerative colitis.

Methods Ten patients with active ulcerative colitis, defined by a Powell-Tuck activity score greater than four points, received a single 3mg/kg natalizumab infusion. The primary end-point was the change in Powell-Tuck score at two weeks post-infusion. Patients were followed-up for 12 weeks post-treatment.

Results Five of the 10 patients achieved a good clinical response at two weeks and one further patient by four weeks, defined by a Powell-Tuck score of less than six points. The median Powell-Tuck scores at one and two weeks post-treatment (7.5 and 6) were lower than the median pre-treatment score of 10. Rescue medication was required by two (20%), three (30%) and eight patients (80%) by weeks two, four and eight, respectively. One patient remained in remission at 12 weeks. The median C-reactive protein (CRP) at two weeks (6mg/l) was lower than that pre-treatment (16mg/l).

Conclusions A single 3mg/kg infusion of natalizumab is well-tolerated in patients with active ulcerative colitis and merits further study as a potential therapy.
5.2 Introduction

As discussed in Chapter 2.3, integrins are hetero-dimeric proteins, consisting of $\alpha$ and $\beta$ subunits, which are important mediators of cell adhesion {Springer 1994}. Immunohistochemistry {Farstad, Halstensen, et al. 1997} and flow-cytometry studies {Künne, Schafer, et al. 1997} {Meenan, Spaans, et al. 1997} suggest that $\alpha 4$ integrin expression is up-regulated on the surface of lymphocytes present in inflamed areas of gut mucosa of ulcerative colitis patients and hence may represent a useful therapeutic target. Monoclonal antibodies to the $\alpha 4$ subunit, either alone or in combination with $\beta 7$ integrin, have been shown to inhibit migration of lymphocytes to rat mesenteric lymph nodes and Peyer's patches in vivo {Issekutz 1991} and to ameliorate colitis in the spontaneously colitic cotton-top tamarin, an animal model of inflammatory bowel disease {Podolsky, Lobb, et al. 1993} {Hesterberg, Winsor-Hines, et al. 1996}. A randomized placebo-controlled study of Act-1, a monoclonal antibody to $\alpha 4\beta 7$ integrin, has demonstrated promising efficacy in patients with active ulcerative colitis, although larger confirmatory studies of this antibody are awaited {Feagan, McDonald, et al. 2000}.

Natalizumab (Antegren™, Elan Pharmaceuticals Inc., South San Francisco, California, USA) is a recombinant humanised antibody to $\alpha 4$ integrin. Preclinical and phase I studies of this antibody have been described in Chapter 2.4, whilst a pilot study of natalizumb in Crohn's disease patients has been described in the preceding chapter. The aim of the following open study was to assess the safety and efficacy of natalizumab in patients with active ulcerative colitis.

5.3 Methods

5.3.1 Patients

Patients with active ulcerative colitis, whose Powell-Tuck score (see Appendix II) was greater than four points {Powell-Tuck, Bown, et al. 1978}, were assessed for trial eligibility. The disease was required to have been diagnosed at least three months prior to trial entry and to have been confirmed by at least two of the following diagnostic criteria: clinical history, histology, radiology or endoscopy.
Female participants were required to have a negative pregnancy test at study entry and to use effective contraception throughout the study.

Patients receiving oral corticosteroids (dose equivalent to \( \leq 40\)mg prednisolone daily) or 5-aminosalicylic acid-derived drugs (dose \( \leq 2.4\)g daily) were included, provided that the dose had not been increased within two weeks of study entry. Likewise, patients receiving azathioprine (dose \( \leq 2\)mg/kg daily) were eligible, provided that this had not been commenced or the dose increased within four months of study entry.

### 5.3.2 Exclusion criteria

The following groups were not included in the study; inpatients, patients who had undergone or who were thought likely to require intestinal surgery up to three months prior to, or three months after study entry, respectively, patients with proctitis only, patients receiving cyclosporin, methotrexate or tacrolimus therapy, patients with an ileostomy, colostomy or ileal pouch, patients with laboratory-confirmed intestinal infection and patients known to have had malignant neoplasia at any site. Patients weighing more than 100kg, and pregnant or breast-feeding women, were also excluded.

### 5.3.3 Design

Patients were assessed for trial eligibility at an initial screening visit, at which the following screening investigations were performed: full blood count, erythrocyte sedimentation rate (ESR), biochemical screen including immunoglobulins and C-reactive protein (CRP), urinalysis, resting electrocardiogram and sigmoidoscopic examination including rectal biopsy. The proportion of peripheral blood T cells (TCR\(\alpha\beta\)+) and B cells (CD19+) were also measured using fluorescence-activated cell-sorter (FACS) analysis (Becton Dickinson, Oxford, UK) \(\{\)Amlot, Tahami, et al. 1996\(\}\). The study was approved by the ethics committee of the Royal Free Hampstead NHS Trust and all patients gave written informed consent prior to participating in the study.

Natalizumab was formulated in a solution of 10mM phosphate-buffered 0.02% polysorbate 80, adjusted to pH 6 with hydrochloric acid and was administered in
100ml of 0.9% saline. Patients received a single 3mg/kg intravenous infusion over 25 to 45 minutes and were observed for at least six hours post-infusion. The Powell-Tuck score was calculated, based on patients’ symptoms and examination findings, at one, two, four, eight and 12 weeks after treatment. A good clinical response was defined as a Powell-Tuck score of less than six points.

Sigmoidoscopic examination and rectal biopsies for histology were taken at each follow-up visit. Appearance of the rectal mucosa at sigmoidoscopy was scored as follows; normal=0, granular mucosa=1, 2=contact bleeding / spontaneous haemorrhage, 3=severe changes; ulceration / severely haemorrhagic mucosa and pus present, {Baron, Connell, et al. 1964}. Biopsies were scored histologically as described by Saverymuttu et al {Saverymuttu, Camillieri, et al. 1986}. Quality of life was assessed by the Inflammatory Bowel Disease Questionnaire (IBDQ) at screening and four weeks after treatment {Guyatt, Mitchell, et al. 1989}. Venous blood was taken at each visit for the haematological, biochemical and FACS analyses described above and for measurement of serum natalizumab and anti-natalizumab antibody levels.

5.3.4 Statistical analysis
Differences between pre-treatment and two-week Powell-Tuck scores, CRP, ESR, platelet counts and albumin concentrations were analysed by the Wilcoxon signed rank test. The study’s primary end-point was the change in Powell-Tuck score at two weeks. The proportion of patients who required ‘rescue medication’ at two weeks was a secondary end-point. This was defined as the initiation or increased daily dose of any of the following drugs, by any route of administration: corticosteroids, 5-aminosalicylates or azathioprine / 6-mercaptopurine. Spearman correlation tests were used to evaluate whether any of the following clinical factors predicted response to natalizumab, as defined by change in Powell-Tuck score at two weeks: pre-infusion Powell-Tuck score, change in CRP at week two, age and disease duration.
5.4 Results

Five of the 15 screened patients did not enter the study because of failure to meet disease activity criteria on the day of treatment (one patient) or personal reasons (four patients). The clinical characteristics of the 10 patients who received natalizumab are shown in Table 5.1, nine of whom completed the post-treatment follow-up to 12 weeks. One patient was withdrawn from the study at two weeks post-infusion on account of severe disease requiring urgent colectomy.

Table 5.1 Demographic characteristics of ulcerative colitis patients at study-entry

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age/ yrs</th>
<th>Disease duration/ months</th>
<th>Powell-Tuck score</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>24.8</td>
<td>23</td>
<td>8</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>49.8</td>
<td>54</td>
<td>8</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>45.8</td>
<td>351</td>
<td>12</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>28.9</td>
<td>62</td>
<td>10</td>
<td>Mesalazine po + pr Azathioprine po Nil</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>41.2</td>
<td>53</td>
<td>8</td>
<td>Nil</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>37</td>
<td>30</td>
<td>11</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>25.5</td>
<td>49</td>
<td>8</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>50.2</td>
<td>79</td>
<td>11</td>
<td>Prednisolone po + pr</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>56.7</td>
<td>152</td>
<td>10</td>
<td>Mesalazine po</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>44.3</td>
<td>63</td>
<td>10</td>
<td>Mesalazine po Prednisolone pr</td>
</tr>
<tr>
<td>Median</td>
<td>42.7</td>
<td>59</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.4.1 Disease activity

The median Powell-Tuck score of 6.0 points at two weeks post-treatment was significantly less than the baseline median score of 10.0 points (p=0.004). A good clinical response (Powell-Tuck <6 points) was achieved in five of the 10 patients at two weeks post-treatment, and in one further patient by week four (Figure 5.1). The mean decrease in the Powell-Tuck score was 4.0 and 4.8 points at weeks two and four, respectively.
Rescue medication was required by two (20%), three (30%) and eight patients (80%) by weeks two, four and eight, respectively (median 34 days; range 8-43). Two of the eight patients rescued at week eight initially demonstrated a good clinical response (Powell-Tuck <6) but experienced a subsequent return of their symptoms. No significant correlation was found between response to natalizumab at week two and age, disease duration, change in CRP at week two or pre-treatment activity score.

**Figure 5.1** The effects of natalizumab on Powell-Tuck score in 10 patients with active ulcerative colitis.

![Graph showing Powell-Tuck score over weeks post-natalizumab](image)

**Figure 5.1** Powell-Tuck activity scores of 10 ulcerative colitis patients after single 3mg/kg natalizumab infusion. Boxes around data-points indicate time at which rescue medication was commenced.
5.4.2 Adverse events
Adverse events were infrequent and were generally thought to be unrelated to natalizumab. The most commonly reported events were 'aggravated ulcerative colitis', representing either a worsening or return of disease symptoms (6 patients), headache, vomiting, lethargy and sore throat, each of which were reported by 2 patients. Three patients required hospital admission during the study, one of whom had failed to respond to natalizumab and required colectomy at two weeks despite rescue with oral and intravenous corticosteroids (patient 10). A second patient (patient 4) was admitted with Campylobacter jejuni enteritis, likely to have been food-borne, 28 days post-infusion and a third (patient 5) developed nausea, vomiting and rigors after commencing oral azathioprine 53 days post-infusion. None of these admissions were assessed as being related to natalizumab.

5.4.3 Haematological and biochemical indices
The median CRP at two weeks post-infusion of 6mg/l was significantly less than that of 16mg/l at baseline (Figure 5.2), but serum albumin and ESR values were unchanged at one and two weeks post-infusion. No significant changes occurred in haemoglobin, platelet counts or serum biochemistry (except CRP) post-infusion with natalizumab.

5.4.4 Drug levels
The mean serum half-life of natalizumab in the ten patients treated was 3.8 days. The mean serum concentrations of natalizumab achieved during the first four weeks post-infusion are shown in Figure 5.3. At eight weeks the serum concentration of natalizumab was negligible (<0.05μg/ml) in all patients. Low-titre anti-idiotypic antibodies (3.4 - 16μg/ml.) to natalizumab developed transiently in one patient (patient 6) and were detected at weeks four, eight and twelve.
Figure 5.2 CRP and ESR values post-natalizumab in 10 ulcerative colitis patients

![Graph showing CRP and ESR values over weeks post-natalizumab.](image)

* p<0.05 compared to baseline

Figure 5.2 Median CRP and ESR values post 3mg/kg natalizumab. Bars are SD.

Figure 5.3 Mean serum concentrations of natalizumab in 10 patients with active ulcerative colitis.

![Graph showing serum natalizumab concentrations over days post-infusion.](image)

mean t1/2=3.8 days (SD 1.5)

Figure 5.3 Serum natalizumab concentrations after 3mg/kg infusion. Bars are SD.
5.4.5 FACS analysis of peripheral blood lymphocytes

A significant increase in total lymphocyte counts occurred post-infusion of natalizumab (Figure 4), from 1.66 +/- 1.03 (mean +/- SD) pre-infusion to 3.32 +/- 1.07, 2.88 +/- 0.78 and 2.01 +/- 0.62 at one, two and four weeks, respectively (Figure 5.4). FACS analyses revealed that this increase was due a rise in both B cell (CD19+) and T cell counts (TCRaβ+). Changes in total lymphocyte count did not correlate with clinical response to natalizumab, as shown in Table 5.2, below.

**Figure 5.4** Lymphocyte counts post-natalizumab in 10 ulcerative colitis patients

![Figure 5.4](image)

**Figure 5.4** Mean lymphocyte counts after 3mg/kg natalizumab infusion. Bars are SD and ** (p<0.005) and * (p<0.05) indicate significant changes compared to baseline.

**Table 5.2** Spearman correlation between change in Powell-Tuck score* and change in total lymphocyte counts in 10 ulcerative colitis patients.

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman r</td>
<td>0.38</td>
<td>-0.15</td>
<td>-0.11</td>
</tr>
<tr>
<td>P value</td>
<td>0.3</td>
<td>0.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Recorded data used for these analyses
5.4.6 Other findings

The median IBDQ quality of life score improved significantly from 101 at baseline to 162 at four weeks post-infusion (p=0.02) in those eight patients with complete data-sets (Figure 5.5; range of possible scores 32 to 224). There were no significant differences in sigmoidoscopic scores following natalizumab treatment (Table 5.3), nor were significant histological changes observed between pre-treatment rectal biopsies (mean score 6.0 +/- 3.0) and those performed at weeks one, two or four (6.2 +/- 2.2, 6.3 +/- 2.0 and 5.9 +/- 3.5, respectively).

**Figure 5.5** Quality of life in ulcerative colitis patients after natalizumab

![Graph showing IBDQ quality of life scores](image)

**Table 5.3** Sigmoidoscopic scores of 10 patients who received natalizumab

<table>
<thead>
<tr>
<th>No</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 8</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>NA</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>NA</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

0=normal, 1=granular mucosa, 2=contact/spontaneous bleeding, 3=severe changes +/- ulceration. Red text indicates rescue therapy commenced.
5.5 Conclusions

The results of this study show that natalizumab is safe and well-tolerated by patients with active ulcerative colitis. The numbers of patients are too small in this open study to assess evidence of efficacy reliably, but the trend to a response in six patients during the first four weeks post-treatment is encouraging. Significant improvements in CRP at two weeks and in quality of life scores at four weeks also support these findings.

Natalizumab’s mean serum half-life of 3.8 days in this study was considerably shorter than the 8.9 days predicted by healthy volunteer studies (Athena 1996), and is consistent with the findings in Crohn’s disease patients, described in the previous chapter. Despite natalizumab’s short half-life in ulcerative colitis patients, a significant increase in the number of circulating peripheral blood lymphocytes was sustained to at least four weeks post-infusion, suggesting that lymphocyte trafficking had been affected. Once again, these findings are consistent with the effects of natalizumab in Crohn’s disease (see Chapter 4).

As mentioned in the previous chapter, further in vitro work has suggested that a minimum serum natalizumab concentration of 5mcg/ml is required to saturate at least 80% of circulating membrane-bound α4 integrins. A concentration of at least this value was sustained to two weeks in just three of the nine patients for whom these data were available. Thus although lymphocyte dynamics were altered by natalizumab, heightened blockade of α4 integrins may be necessary to improve efficacy and further studies are required to assess the optimal dose and dose interval in ulcerative colitis patients.

Although this small study demonstrates a trend to efficacy for natalizumab, some patients did not respond, notably patient 10, in spite of experiencing an increase in circulating lymphocytes. Furthermore, the percentage increase in peripheral blood lymphocytes at each time-point did not correlate with patients’ individual clinical responses to natalizumab overall (Table 5.2), suggesting that factors other than α4 integrins may be important in perpetuating mucosal inflammation in the human gut. The lack of demonstrable change in histological parameters post-infusion is disappointing, but perhaps predictable in the light of the pharmacokinetic findings.
In summary, this study demonstrates that natalizumab (Antegren™), an anti-α4 integrin antibody is well-tolerated in patients with active ulcerative colitis. There is a positive trend to efficacy, but the present dosing regimen is sub-optimal. A larger randomised placebo-controlled trial is necessary to establish the efficacy of natalizumab and hence its potential role in the treatment of ulcerative colitis.
Chapter 6

The effects of natalizumab on peripheral blood leucocyte subsets

6.1 Abstract

Introduction and aims

Alpha-4 integrins are known to be widely expressed on all leucocyte subtypes except neutrophils. This study aimed to measure the effects of a single 3mg/kg infusion of natalizumab, a humanised monoclonal antibody to α4 integrin, on basic leucocyte subsets, T cells expressing NK cell and activated T cells in patients with active IBD.

Methods

Venous blood samples were taken from IBD patients who had entered the studies described in Chapters 4 and 5. Samples were taken prior to and at one, two, four, eight and twelve weeks after natalizumab infusion. Basic leucocyte subsets and specific T cell markers were measured at each time-point in addition to serial serum natalizumab levels and disease activity scores.

Results

Eosinophil, monocyte, B and T cell counts were significantly increased for at least one week post-natalizumab in IBD patients. Neutrophil and basophil counts were unchanged. T cells expressing CD25, CD26, HLA-DR, CD8DR, CD8CD28, CD45RO and CD45RO were all significantly increased compared to baseline to at least week four. T cells expressing CD38 and CD69 were also increased, but the effect was less sustained in Crohn’s disease patients. NK cells (CD16) were unchanged post-infusion in all patients and NK-type T cells (CD57) were increased at week one in Crohn’s disease patients only. Gamma-delta T cells were not affected by natalizumab in either patient group. T cells expressing monoclonal kappa light chains were significantly elevated at to at least four weeks, but values did not correlate with serum natalizumab level. Changes in other lymphocyte subsets did not correlate with disease activity or serum natalizumab levels.

Conclusions

A single 3mg/kg natalizumab infusion produced increased circulating levels of most leucocyte subsets except neutrophils, basophils and NK cells for at least four weeks, suggesting that leucocyte trafficking had been interrupted.
6.2 Introduction and aims

Previous studies have demonstrated that natalizumab produces a sustained rise in circulating peripheral blood leucocytes in animals and healthy volunteers, as described in 2.4.3. It is not clear from these early studies, however, whether natalizumab could have differential effects on leucocyte subsets, given that all leucocytes except neutrophils express α4 integrins. The studies described in this chapter aimed to test the hypothesis that α4 integrin blockade by natalizumab increases circulating numbers of leucocyte subsets involved in the pathogenesis of IBD, namely activated T cells, monocytes, eosinophils, natural killer cells and γδ+ T cells.

6.3 Methods

6.3.1 Patients

Samples were taken from 10 patients with active ulcerative colitis (all received natalizumab) and 30 Crohn's disease patients (18 natalizumab, 12 placebo) who had been enrolled in the trials described in Chapters 4 and 5. Venous blood was taken immediately prior to natalizumab/placebo infusion and at one, two, four, eight and 12 weeks post-infusion.

6.3.2 Leucocyte analyses

Analyses of basic leucocyte subgroups (lymphocytes, neutrophils, basophils, monocytes, eosinophils) were performed on EDTA-preserved samples using a standard Coulter-counter method. The LymphoprepTM technique (Nycomed, Denmark) was used to isolate peripheral blood lymphocytes (PBLs) from lithium-heparinised venous blood samples prior to analysis by multi-colour fluorescence-activated cell sorter (FACS; Becton Dickinson, Oxford, U.K.) in conjunction with Consort 30 software, as described previously {Amlot, Tahami, et al. 1996}. Samples were stored for a maximum of 24 hours at room temperature prior to PBL separation. The percentages of PBLs expressing the following markers were measured: CD19 (B cell), TCRαβ (T cell), CD3 (pan-T cell), TCRγδ (T cell), CD4 (helper/Th-1 T cell), CD8 (cytotoxic/suppressor T cell) and CD16 (NK cell). The percentages of TCRαβ+ cells expressing the activation antigens CD38, CD25 (α chain of the interleukin-2 receptor), CD26, CD69 and HLA-DR were measured, in addition to naïve (CD45RA+) and memory (CD45RO+) T cell
subsets and ‘NK-T cells’ (CD57+CD3+). The percentage of cytotoxic/suppressor T cells (CD8+) expressing the activation antigens CD28 and HLA-DR were also measured. Finally, the proportion of TCRαβ+ cells expressing monoclonal kappa light chains were measured as an indicator of natalizumab binding to leucocytes. (see Appendix III for monoclonal antibody suppliers’ details).

6.3.3 Other parameters
At each visit, disease activity was measured using the CDAI or Powell-tuck colitis score and serum was stored for measurement of natalizumab levels, as described in Chapters 4 and 5.

6.3.4 Statistical analysis
Wilcoxon signed rank test was used for analysis of changes in leucocyte subsets compared to baseline within treatment groups, p<0.05 denoting significance. Kruskal-Wallis test was used for between group analyses, with Dunn’s post-test correction. Spearman rank correlation tests were used to assess correlation between disease activity parameters, natalizumab levels and leucocyte subsets in patients who received natalizumab only, at one, two and four weeks post-infusion.

6.4 Results
6.4.1 Basic leucocyte subsets
Patients’ median ages (years) were as follows: Crohn’s disease (natalizumab-treated) 31.6, Crohn’s disease (placebo-treated) 33.7 and ulcerative colitis 42.7. Total lymphocyte counts, both B and T cells, were significantly increased compared to baseline values following natalizumab infusion (Figures 4.6 and 5.4 in previous chapters). Lymphocyte counts remained significantly elevated at four weeks post-infusion in both Crohn’s patients (p=0.002) and in those with ulcerative colitis (p=0.02), before returning to pre-treatment values at week 8. These results have been described in Chapters 4 and 5, respectively. There was a significant increase in eosinophils in Crohn’s disease and ulcerative colitis patients following natalizumab, which persisted to at least four weeks in the Crohn’s group (p=0.02 at four weeks compared to baseline) and to one week only in the ulcerative colitis group (p=0.02; Fig. 6.1a). Additionally, monocyte counts increased significantly compared to baseline at one and two weeks in the
ulcerative colitis group (p=0.02, both time-points) and one week only in those with Crohn’s disease (p=0.03; Fig. 6.1b). Neutrophil and basophil counts remained unchanged in both studies. (see Table 6a)

6.4.2 T cells expressing activation antigens

6.4.2.1 Crohn’s disease patients

TCRαβ+ cells expressing the activation markers CD45RA, CD45RO, CD26 and HLA-DR were significantly increased compared to baseline at one, two and four weeks post-infusion. CD25+ cells were significantly increased at four weeks only. Activated CD8+ cells expressing CD28 and HLA-DR were also significantly elevated at one and two weeks post infusion, but only those expressing CD28 remained elevated at four weeks post-infusion. T cells expressing CD38 and CD69 were unchanged at all time-points post-natalizumab infusion (Fig. 6.2a and 6.2c).

6.4.2.2 Ulcerative colitis patients

The effects of natalizumab in ulcerative colitis patients broadly paralleled those in Crohn’s disease patients. At one week post-infusion, CD45RA+, CD45RO+, CD25+, CD26+, HLA-DR+, CD8CD28+ and CD8DR+ cells were all significantly increased. By contrast with the Crohn’s group, T cells expressing CD38 and CD69 were also significantly elevated post-infusion, CD69+ cells being increased to at least two weeks, whilst CD38+ cells were increased at one week only. In both groups, counts of T cells expressing activation antigens had returned to pre-treatment values by eight weeks for all subsets (Fig. 6.2b and Fig. 6.2d).
Figure 6.1a The effect of natalizumab on circulating eosinophils in patients with active Crohn’s disease and ulcerative colitis.

Figure 6.1a Significantly increased circulating eosinophil counts in Crohn’s disease (n=18) and ulcerative colitis patients (n=12) after 3mg/kg natalizumab infusion.

Figure 6.1b The effect of natalizumab on circulating monocytes in patients with active Crohn’s disease and ulcerative colitis.

Figure 6.1b Significantly increased monocyte counts in active Crohn’s disease and ulcerative colitis patients after 3mg/kg natalizumab infusion.
Figure 6.2a The effects of natalizumab on TCRαβ+ cells expressing activation antigens in patients with active Crohn's disease.

![Graph](image)

- CD8CD28
- CD8DR
- CD25
- CD26
- DR

* p<0.05; ** p<0.005 compared to baseline

Figure 6.2a Significantly increased TCRαβ+ cells expressing CD26, HLA-DR, CD8DR and CD8CD28 to at least four weeks post 3mg/kg natalizumab infusion.

---

Figure 6.2b The effects of natalizumab on TCRαβ+ cells expressing activation antigens in patients with active ulcerative colitis.

![Graph](image)

- CD8CD28
- CD8DR
- CD25
- CD26
- DR

* p<0.05; ** p<0.005 compared to baseline

Figure 6.2b Significantly increased TCRαβ+ cells expressing CD26, HLA-DR, CD8DR and CD8CD28 to at least two weeks post 3mg/kg natalizumab infusion.
Figure 6.2c  The effects of natalizumab on TCRαβ+ cells expressing
activation antigens and memory and naïve markers in patients with active
Crohn’s disease

![Graph showing changes in TCRαβ+ cell counts during weeks post-natalizumab infusion.](image)

*Figure 6.2c*  Significantly increased memory (CD45RO) and naïve (CD45RA) TCRαβ+ cells to at least four weeks post-natalizumab infusion in Crohn’s disease patients.

Figure 6.2d  The effects of natalizumab on TCRαβ+ cells expressing
activation antigens, memory and naïve markers in patients with active
ulcerative colitis.

![Graph showing changes in TCRαβ+ cell counts during weeks post-natalizumab infusion.](image)

*Figure 6.2d*  Significantly increased memory (CD45RO), naïve (CD45RA), CD69 and CD38 TCRαβ+ cells at one week after 3mg/kg natalizumab infusion in ulcerative colitis patients.
Figure 6.3a The effects of natalizumab on circulating TCRγδ+ and NK-type cells in patients with active Crohn’s disease.

![Graph showing the effects of natalizumab on circulating TCRγδ+ and NK-type cells in Crohn's disease patients.](image)

Weeks post-natalizumab

- CD57CD3+
- CD16CD3-
- gamma-delta cells

* p<0.05 compared to baseline

Figure 6.3b The effects of natalizumab on circulating TCRγδ+ and NK-type cells in patients with ulcerative colitis.

![Graph showing the effects of natalizumab on circulating TCRγδ+ and NK-type cells in ulcerative colitis patients.](image)

Weeks post-natalizumab

- CD57+CD3+
- CD16+CD3-
- gamma-delta cells

* p<0.05 compared to baseline

**Figures 6.3a & 6.3b:** Overall lack of significant effect of 3mg/kg natalizumab infusion on TCRγδ+ and NK-type cells in patients with active IBD
6.4.3 Natural killer-type cells (NK cells) and gamma-delta T cells

NK cell counts (CD16+CD7-) were unchanged after natalizumab in Crohn’s disease patients at all time-points and raised at week four only in ulcerative colitis patients. Additionally, NK-T cells (CD57+CD3+) were unchanged in ulcerative colitis patients at all time-points and significantly elevated in Crohn’s disease patients at one week post-infusion only (p=0.007). γδT cell counts were not affected by natalizumab in either treatment group (Figs. 6.3a and 6.3b).

6.4.4 T cells expressing monoclonal kappa light chains

Counts of TCRαβ cells expressing monoclonal kappa light chain were significantly elevated at one and two weeks post-natalizumab in both groups, and remained significantly elevated at four weeks in the Crohn’s disease group only (Figure 6.4).

Figure 6.4 The effect of natalizumab on monoclonal kappa light chain+ TCRαβ+ cells in patients with active Crohn’s disease (n=18) and ulcerative colitis (n=10).

```
* p<0.05; ** p<0.005 compared to baseline
```

Figure 6.4 Significant elevation in TCRαβ+ kappa light chain cells after natalizumab.
Table 6a  Leucocyte subsets in placebo-treated Crohn's disease patients

<table>
<thead>
<tr>
<th>Subset</th>
<th>Week 0 Mean SD</th>
<th>Week 1 Mean SD</th>
<th>Week 2 Mean SD</th>
<th>Week 4 Mean SD</th>
<th>Week 8 Mean SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>0.60 0.33</td>
<td>0.55 0.36</td>
<td>0.82 0.67</td>
<td>0.48 0.29</td>
<td>0.51 0.31</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7.21 2.75</td>
<td>8.18 3.48</td>
<td>7.86 3.36</td>
<td>7.61 2.26</td>
<td>6.91 2.15</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.19 0.16</td>
<td>0.17 0.13</td>
<td>0.15 0.13</td>
<td>0.20 0.12</td>
<td>0.14 0.10</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.19 0.30</td>
<td>0.14 0.09</td>
<td>0.09 0.07</td>
<td>0.09 0.03</td>
<td>0.08 0.05</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.60 0.33</td>
<td>0.55 0.36</td>
<td>0.82 0.67</td>
<td>0.48 0.29</td>
<td>0.51 0.31</td>
</tr>
</tbody>
</table>

Table 6a  Mean (SD) leucocyte subset values post-placebo; no significant differences in any group compared to baseline (week 0) values.

Table 6b  T cell and NK markers in placebo-treated Crohn's disease patients

<table>
<thead>
<tr>
<th>Subset</th>
<th>Week 0 Mean SD</th>
<th>Week 1 Mean SD</th>
<th>Week 2 Mean SD</th>
<th>Week 4 Mean SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>1.36 0.57</td>
<td>1.03 0.51</td>
<td>1.25 0.79</td>
<td>0.93 0.37</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>0.99 0.53</td>
<td>0.73 0.52</td>
<td>0.78 0.47</td>
<td>0.65 0.38</td>
</tr>
<tr>
<td>CD25+</td>
<td>0.18 0.11</td>
<td>0.10 0.05</td>
<td>0.13 0.08</td>
<td>0.10 0.06</td>
</tr>
<tr>
<td>CD26+</td>
<td>0.49 0.32</td>
<td>0.34 0.24</td>
<td>0.43 0.32</td>
<td>0.32 0.20</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>0.73 0.41</td>
<td>0.52 0.36</td>
<td>0.55 0.30</td>
<td>0.44 0.26</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.42 0.20</td>
<td>0.28 0.14</td>
<td>0.39 0.26</td>
<td>0.27 0.11</td>
</tr>
<tr>
<td>CD38+</td>
<td>0.42 0.27</td>
<td>0.26 0.19</td>
<td>0.28 0.22</td>
<td>0.24 0.15</td>
</tr>
<tr>
<td>CD69+</td>
<td>0.14 0.20</td>
<td>0.07 0.07</td>
<td>0.17 0.15</td>
<td>0.05 0.05</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>0.19 0.10</td>
<td>0.14 0.08</td>
<td>0.21 0.19</td>
<td>0.13 0.06</td>
</tr>
<tr>
<td>CD8+</td>
<td>0.35 0.14</td>
<td>0.26 0.12</td>
<td>0.42 0.23</td>
<td>0.23 0.08</td>
</tr>
<tr>
<td>CD8+CD28+</td>
<td>0.21 0.11</td>
<td>0.15 0.11</td>
<td>0.19 0.13</td>
<td>0.13 0.08</td>
</tr>
<tr>
<td>CD8+DR+</td>
<td>0.11 0.09</td>
<td>0.09 0.06</td>
<td>0.16 0.17</td>
<td>0.08 0.05</td>
</tr>
<tr>
<td>CD57+CD3+</td>
<td>0.08 0.06</td>
<td>0.05 0.05</td>
<td>0.10 0.09</td>
<td>0.07 0.04</td>
</tr>
<tr>
<td>CD16+CD3-</td>
<td>0.11 0.09</td>
<td>0.11 0.09</td>
<td>0.15 0.19</td>
<td>0.08 0.04</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.11 0.16</td>
<td>0.08 0.10</td>
<td>0.15 0.19</td>
<td>0.08 0.04</td>
</tr>
<tr>
<td>KappaMAb+</td>
<td>0.22 0.24</td>
<td>0.33 0.49</td>
<td>0.39 0.57</td>
<td>0.83 0.83</td>
</tr>
</tbody>
</table>

Table 6b  Columns show mean counts (SD) of T cell subsets and NK-type cells of Crohn’s study placebo patients compared with baseline values (Wilcoxon signed rank test; p<0.05). Significant differences compared to baseline are shown in bold. Patients who received rescue corticosteroids were not included in the analysis from the point at which treatment commenced.

6.4.5 Placebo group patients

Results of leucocyte analyses were available for 11 patients. No significant changes in basic leucocyte subsets were detected following natalizumab infusion (Table 6a). Some changes in lymphocytes expressing activation antigens were detected, although these occurred at week one only (Table 6b). Counts of
TCRαβ+ lymphocytes expressing CD45RO, CD45RA, CD38 and HLA-DR were all significantly decreased compared to baseline at one week post-infusion (p=0.04, p=0.02, p=0.03, p=0.04, respectively). In addition, CD8+ cell counts were also significantly reduced compared to baseline (p=0.049).

6.4.6 Correlation of leucocyte subsets with other parameters
There was no significant correlation between total lymphocyte counts and disease activity score in either Crohn’s disease or ulcerative colitis patients (Table 6c), nor were any significant correlations found between individual lymphocyte subsets and disease activity. Similarly, no significant correlation was detected between serum natalizumab level and change in leucocyte subsets at one, two or four weeks post-infusion (Table 6d). Finally, no significant correlation was found between natalizumab levels and counts of TCRαβ+ cells expressing kappa light chains at these time-points (Table 6d). Natalizumab was undetectable in almost all patients at eight weeks, thus correlations were not calculated for this time-point.

Table 6c Spearman r values comparing lymphocyte subsets and disease activity

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.16</td>
<td>-0.37</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>0.26</td>
<td>-0.29</td>
</tr>
<tr>
<td>CD25+</td>
<td>0.44</td>
<td>0.07</td>
</tr>
<tr>
<td>CD26+</td>
<td>0.31</td>
<td>-0.22</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>0.29</td>
<td>-0.25</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.32</td>
<td>-0.27</td>
</tr>
<tr>
<td>CD38+</td>
<td>0.31</td>
<td>-0.08</td>
</tr>
<tr>
<td>CD69+</td>
<td>0.32</td>
<td>0.12</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>0.19</td>
<td>-0.05</td>
</tr>
<tr>
<td>CD8+CD28+</td>
<td>0.01</td>
<td>-0.17</td>
</tr>
<tr>
<td>CD8+DR+</td>
<td>0.11</td>
<td>-0.19</td>
</tr>
<tr>
<td>CD57+</td>
<td>0.19</td>
<td>-0.08</td>
</tr>
<tr>
<td>CD16+</td>
<td>-0.08</td>
<td>-0.03</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.34</td>
<td>0.14</td>
</tr>
<tr>
<td>Kappa Mab+</td>
<td>-0.11</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

Table 6c No significant correlation found between lymphocyte subset counts and CDAI (Crohn’s disease patients) or Powell-Tuck score (ulcerative colitis).
### Table 6d

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.03</td>
<td>0.49</td>
</tr>
<tr>
<td>TCR6β+</td>
<td>-0.11</td>
<td>0.35</td>
</tr>
<tr>
<td>CD25+</td>
<td>-0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>CD26+</td>
<td>-0.07</td>
<td>0.28</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>0.19</td>
<td>0.29</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>-0.16</td>
<td>0.32</td>
</tr>
<tr>
<td>CD38+</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>CD69+</td>
<td>0.02</td>
<td>0.26</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>CD8+CD28+</td>
<td>-0.18</td>
<td>0.16</td>
</tr>
<tr>
<td>CD8+DR+</td>
<td>0.4</td>
<td>0.16</td>
</tr>
<tr>
<td>CD57+</td>
<td>0.1</td>
<td>-0.06</td>
</tr>
<tr>
<td>CD16+</td>
<td>0.22</td>
<td>0.19</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.002</td>
<td>-0.16</td>
</tr>
<tr>
<td>Kappa Mab+</td>
<td>-0.07</td>
<td>-0.27</td>
</tr>
</tbody>
</table>

No significant correlation between disease activity and lymphocyte subsets, except for total lymphocyte counts at week 4 (p=0.04).

### 6.5 Conclusions

A single 3mg/kg natalizumab infusion produced increased circulating levels of most leucocyte subsets in patients with active IBD, suggesting that trafficking had been interrupted. Circulating eosinophil, monocyte and lymphocyte counts were significantly elevated above baseline values for at least four weeks post-infusion in most patients. This is interesting, in that serum natalizumab levels had fallen below that needed to produce blockade of at least 80% α4 positive leucocytes and suggests that natalizumab’s activity persisted in spite of low plasma concentrations.

The proportion of a wide range of circulating T cell subsets were significantly increased above pre-treatment values, particularly those expressing activation antigens. The changes paralleled those of total lymphocyte counts. Lymphocytes expressing the γδ T cell receptor were not affected by natalizumab, however, suggesting that α4 integrins are either not expressed, or are expressed at lower levels on these cells. NK cells (CD16+CD3-) were similarly unaffected and notably CD57+ T cells were affected to a much lesser extent by natalizumab than other T cell subsets. The results of this chapter will be discussed further in Chapter 9, section 9.5.3.
Chapter 7
Leucocyte and serum adhesion molecule expression in IBD.

7.1 Abstract

Introduction
Serum concentrations of the soluble adhesion molecules may be related to IBD activity. This study aimed to examine the effects of monoclonal antibody blockade of α4 integrins on serum and circulating lymphocyte adhesion molecule expression in patients with active IBD.

Methods
Samples were taken from 40 IBD patients (30 Crohn’s disease; 10 ulcerative colitis) with active disease (CDAI>150 or Powell-Tuck colitis score >4) who randomly received a single 3mg/kg natalizumab infusion or placebo. Samples were also taken from 10 healthy volunteers. In IBD patients, serum levels of VCAM-1, ICAM-1 and E-selectin were measured by ELISA at baseline, one and two weeks post-infusion. The numbers of TCRαβ+ cells expressing CD49d, CD54 and CD62L were also measured by FACS analysis.

Results
Mean serum VCAM-1 levels were significantly reduced at one and two weeks compared to baseline in ulcerative colitis (p=0.02; p=0.04) and Crohn’s disease patients (p=0.008; p=0.02). CD49d+ T cell counts were significantly increased at one week in ulcerative colitis patients only (p=0.03). Mean serum ICAM-1 levels were not significantly changed in either ulcerative colitis or Crohn’s disease patients, although CD54+ T cell counts were significantly elevated at one and two weeks in both groups (p<0.01 ulcerative colitis; p=0.03 Crohn’s disease). E-selectin concentrations were significantly increased at two weeks in UC patients only but CD62L+ T cell counts were raised at one week in ulcerative colitis patients (p=0.002) and at two weeks in Crohn’s disease patients (p=0.03). Baseline serum soluble adhesion molecule concentrations of IBD patients did not differ significantly from those of healthy volunteers.

Conclusions
A single 3mg/kg dose of natalizumab produces a significant reduction in serum soluble VCAM-1, an endothelial ligand of α4 integrin, suggesting that this interaction may be down-regulated post-treatment in patients with IBD. Increased
numbers of circulating CD54+ and L-selectin+ T cells suggest that trafficking of these cells is also interrupted but less consistent effects on their soluble ligands were demonstrated.

7.2 Introduction and aims

Soluble adhesion molecules can be detected in the serum of IBD patients, although their precise relationship to disease activity is unclear. To date, the adhesion molecules VCAM-1, ICAM-1 and E-selectin have been evaluated in IBD patients compared with healthy controls {Patel, Pall, et al. 1995}. As discussed in Chapter 2 (section 2.3), it has been proposed that increased serum concentrations of adhesion molecules may represent increased turnover of membrane-bound adhesion molecules generally, since both leucocyte-bound and endothelial adhesion molecules are increased in active IBD.

The principal aim of these studies was to examine the effects of natalizumab-mediated blockade of α4 integrins on serum adhesion molecule concentration and T cell expression of adhesion molecules in patients with active IBD. The monoclonal antibody CD49d was chosen as a 'pan-α4 integrin' marker, i.e. to detect α4 integrins in conjunction with either β1 or β7 integrins. CD54 (ICAM-1) was chosen as a lymphocyte target from which to assess β2 integrin (LFA-1 or CD11aCD18) activity and T cell activation. As described in section 2.3, the interaction between leucocyte β2 integrin and endothelial ICAM-1 is thought to be important in mediating adhesion between these cell types. CD54 (ICAM-1) also exists on the surface of lymphocytes, where it is thought to mediate T lymphocyte activation and adhesion via co-stimulation with lymphocyte β2 integrins (LFA-1) {van de & van der Saag 1996}. Finally the monoclonal antibody, CD62L, was selected as a marker of T cell selectin activity. Additionally, serum and lymphocyte adhesion molecule expression was compared between IBD patients at baseline and that of healthy control volunteers. The hypothesis for these studies was that interruption of α4 integrin-mediated leucocyte adhesion by natalizumab results in interrupted leucocyte trafficking, with subsequent increased proportions of circulating adhesion molecule-expressing leucocytes and changes in serum levels of soluble adhesion molecules.
7.3 Methods

7.3.1 Patients' sera for soluble adhesion molecule assays
Venous blood samples were collected from 15 Crohn’s disease and 10 ulcerative colitis patients who were taking part in the trials of natalizumab (Antegren™; Elan Pharmaceuticals Inc.) at the Royal Free Hospital, London described in Chapters 4 and 5 (i.e. Crohn’s disease patients who entered the study at Royal Gwent Hospital were not included in these analyses). Eight of the 15 Crohn’s disease patients received active drug and seven patients received placebo. All 10 ulcerative colitis patients received active drug. Samples were taken from patients immediately pre-treatment and at one and two weeks post-infusion. All patients had active IBD at baseline, as defined by a CDAI >150 points or Powell-Tuck colitis score >4 points. Samples were also taken from ten healthy medical school staff volunteers. Samples from volunteers found to have elevated CRP, ESR or total white cell counts at the time of the study were discarded, since any of these changes may have represented altered immune profile or co-existent pathology, such as an upper respiratory tract infection.

7.3.2 Assay methods
Sera were stored at -70°C, having been extracted from clotted samples centrifuged at 2500 r.p.m. for 10 minutes. Prior to performing the assays, frozen sera were thawed, mixed thoroughly and lipaemic or grossly haemolysed samples were discarded. Serum concentrations of VCAM-1, ICAM-1 and E-selectin were measured by ELISA using commercial ELISA kits obtained from R&D Systems Abingdon, Oxford, UK (see appendix III for supplier’s details). Working standards, positive and negative control samples were prepared and all samples were diluted with buffered protein base to between 20 and 50-fold, depending on the adhesion molecule being assayed. All samples were assayed in duplicate using a standard 96 micro-welled plate. 100μl of standard, sample or control was added to 100μl diluted conjugate (sheep polyclonal antibody to human serum VCAM-1, ICAM-1 or E-selectin) and incubated at room temperature for 1.5 hours. Each well was then aspirated and washed six times using 300μl of wash buffer (buffered surfactant). 100μl of tetramethylbenzidine (substrate) was added to each well and incubated for a further 20 minutes. 100μl of stop solution (acid solution) was then added to the wells in the same order as the substrate. Each well’s optical
density was determined using a microplate reader set at 450nm, with wavelength correction set at 620nm. The mean absorbance of each well was calculated for each set of duplicate standards. A standard curve of these results was generated using four parametric logistic curve-fit software (Microsoft Excel 97). The concentration of each unknown sample was then calculated using the equation of the curve. Each assay was validated by showing that the concentration of the positive control sample was accurately predicted by each assay’s standard absorbance curve. The coefficient of variation between six pairs of duplicate samples was used to assess intra-assay precision for each ELISA plate.

7.3.3 Patients' blood samples for lymphocyte analyses
Venous blood samples for these analyses were available from all 30 patients (18 natalizumab-treated, 12 placebo) who took part in the Crohn’s disease study described in Chapter 4, i.e. from both Royal Free Hospital and Royal Gwent Hospital sites. Samples were also taken from the 10 patients who took part in the open ulcerative colitis study described in Chapter 5 and from the 10 healthy controls described above.

7.3.4 FACS analysis of lymphocyte adhesion molecules
Peripheral blood lymphocytes were extracted from lithium-heparinised venous samples using the Lymphoprep technique (Nycomed, Denmark). Total circulating lymphocyte counts and the percentage of TCRαβ+ cells expressing α4 integrins (CD49d), ICAM-1 (CD54) and L-selectin (CD62L) were measured by multicolour fluorescence-activated cell-sorter analysis (Becton-Dickinson; Oxford, UK) as described previously {Amlot, Tahami, et al. 1996}. Details of suppliers of monoclonal antibodies are shown in Appendix III.

7.3.5 Statistics
Wilcoxon signed rank test was used to test for significant changes from baseline (p<0.05) within disease groups. Kruskal-Wallis one-way ANOVA with Dunn’s post test was used to test for significant differences between IBD patients at baseline and healthy volunteers.
7.4 Results

7.4.1 Serum adhesion molecules
The median ages of natalizumab-treated Crohn’s disease patients, placebo-treated Crohn’s disease patients, ulcerative colitis patients and controls were 31.3 years, 26.5 years, 42.7 years and 35.7 years, respectively. Crohn’s disease patients were significantly younger than both controls and ulcerative colitis patients (p=0.004 and p=0.005, respectively). Details of intra- and inter-assay precision analyses are shown in Appendix IV. There were no significant differences in serum soluble adhesion molecule concentrations between disease groups and healthy volunteers at baseline (See Fig. 7.1). Mean serum VCAM-1 levels were significantly reduced at one and two weeks after natalizumab infusion compared to baseline in ulcerative colitis (1 week, 2 week values; p=0.02, p=0.04) and Crohn’s disease patients (p=0.008, p=0.02; Fig. 7.2). There were no significant changes detected in soluble ICAM-1 levels post-natalizumab at one or two weeks in either ulcerative colitis or Crohn’s disease patients (Figure 7.3). E-selectin concentrations were significantly increased at two weeks in ulcerative colitis patients only (Figure 7.4). There were no significant differences in the serum concentrations of VCAM-1, ICAM-1 or E-selectin in Crohn’s disease patients who received placebo at one or two weeks compared to baseline (Fig. 7.5).

7.4.2 Lymphocyte studies
Adequate FACS data from healthy volunteers was available for CD49d+ cells only, due to technical problems. Mean values of these cells were significantly lower than baseline Crohn’s values (p<0.01; Fig 7.6), but were not significantly different from those of ulcerative colitis patients. Total lymphocyte counts were significantly increased post-natalizumab treatment in Crohn’s disease and ulcerative colitis patients as described in chapter 4. CD49d+TCRαβ+ cell counts were significantly increased at one week in ulcerative colitis patients (p=0.03), but no significant changes in these cells were found in Crohn’s patients (Figure 7.7) compared to baseline. CD54+TCRαβ+ counts were significantly elevated at one and two weeks in both ulcerative colitis and Crohn’s disease groups (one and two weeks; p<0.01 ulcerative colitis; p=0.03 Crohn’s disease; Figure 7.8). CD62L+TCRαβ+ counts were raised at one week in ulcerative colitis patients (p=0.002) and at two weeks in Crohn’s disease patients (p=0.03; Figure 7.9). In
Crohn’s disease patients who received placebo (n=7), CD54+TCRαβ+ counts were significantly lower than baseline at one week only (p=0.03).

**Figure 7.1** Comparison of serum soluble adhesion molecule concentrations between disease groups at baseline.

---

**Figure 7.1** Graph showing no significant difference between controls, patients with active Crohn’s disease and patients with active ulcerative colitis at baseline. Columns show mean serum adhesion molecule concentrations +/- SD.
Figure 7.2  The effect of natalizumab on serum VCAM-1 concentrations in patients with active Crohn's disease (n=8) and ulcerative colitis (n=10).

![Graph showing the effect of natalizumab on serum VCAM-1 concentrations.](image)

* p≤0.04
* p≤0.02

Figure 7.2  Lines are means +/- SD. * = significance compared to baseline.

Figure 7.3  The effect of natalizumab on serum ICAM-1 concentrations in patients with active Crohn's disease and ulcerative colitis.

![Graph showing the effect of natalizumab on serum ICAM-1 concentrations.](image)

Figure 7.3  No significant changes in serum ICAM-1 concentrations at one and two weeks after 3mg/kg natalizumab infusion in Crohn’s disease (n=8) and ulcerative colitis (n=10) patients.
Figure 7.4  The effect of natalizumab on serum E-selectin in patients with active Crohn’s disease (n=8) and ulcerative colitis (n=10).

Figure 7.4  No significant changes in E-selectin levels in Crohn’s disease or ulcerative colitis patients.

Figure 7.5  Serum soluble adhesion molecule concentrations in Crohn’s disease patients who received placebo.

Figure 7.5  No significant differences found in serum VCAM-1, ICAM-1 or E-selectin concentrations in Crohn’s disease patients who received placebo.
Figure 7.6  Comparison of circulating \( \alpha 4^+ \) TCR\( \alpha\beta \) lymphocyte counts between IBD patients and controls at baseline.

![Graph showing comparison of circulating \( \alpha 4^+ \) TCR\( \alpha\beta \) lymphocyte counts](image)

* \( p=0.04 \)

Figure 7.6  Patients with active Crohn's disease (n=15) had significantly lower baseline CD49d+ cell counts than controls (n=8).

Figure 7.7  The effects of natalizumab on circulating TCR\( \alpha\beta^+ \) lymphocytes which express \( \alpha 4 \) integrins (CD49d+) in patients with active IBD.

![Graph showing effects of natalizumab](image)

* \( p=0.03 \)

Figure 7.7  Significant increase in circulating CD49d+ (\( \alpha 4 \) integrin+) T cells at 1 week after 3mg/kg natalizumab infusion in ulcerative colitis patients compared to baseline. No significant changes in Crohn's patients. Values are means +/- SD.
Figure 7.8 The effects of natalizumab on circulating TCRαβ+ lymphocytes which express ICAM-1 (CD54+) in patients with active IBD.

![Graph showing the effects of natalizumab on circulating TCRαβ+ lymphocytes which express ICAM-1 (CD54+).](image)

**Figure 7.8** Significantly increased peripheral CD54+ TCRαβ+ cells in patients with active IBD at one and two weeks after 3mg/kg natalizumab.

Figure 7.9 The effects of natalizumab on circulating TCRαβ+ lymphocytes which express L-selectin (CD62L+) in patients with active IBD.

![Graph showing the effects of natalizumab on circulating TCRαβ+ lymphocytes which express L-selectin (CD62L+).](image)

**Figure 7.9** Significantly increased peripheral CD62L+ T cells in ulcerative colitis (n=10) and Crohn’s disease patients (n=8) at one and two weeks after a single 3mg/kg natalizumab infusion, respectively. * = p<0.05 compared to baseline.
Figure 7.10  The effects of placebo on circulating TCR\(\alpha\beta^+\) cells expressing CD49d (\(\alpha 4\) integrin), CD54 (ICAM-1) and CD62L (L-selectin) in patients with active Crohn's disease.

<table>
<thead>
<tr>
<th>Weeks post-placebo</th>
<th>CD62L+</th>
<th>CD49d+</th>
<th>CD54+</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* \(p=0.03\)  

Figure 7.10  Significant reduction in CD54+TCR\(\alpha\beta^+\) cells at one week post-placebo only. No significant changes in other T cell subsets.

7.5 Conclusions

Serum adhesion molecule concentrations of patients with active IBD at baseline did not differ significantly from those of healthy volunteers. This finding differs from the work of others, but the number of patients and controls studied above were probably too small to make any firm conclusions. Blockade of \(\alpha 4\) integrins by natalizumab resulted in significant reduction in circulating VCAM-1 levels in both Crohn's disease and ulcerative colitis patients, for at least two weeks post-infusion. This finding suggests that \(\alpha 4\) integrin-mediated leucocyte trafficking is linked in some way to serum soluble VCAM-1 expression and possibly to expression by endothelial cells too. Less consistent results were found for serum soluble ICAM-1 and E-selectin concentrations.

Natalizumab appeared to have minimal effects on circulating T cells expressing \(\alpha 4\) integrins (CD49d+). This somewhat conflicting finding is likely to have been due to FACS-related technical problems, which will be described in the discussion section of Chapter 9. By contrast, IBD patients developed increased ICAM-1+ T cells for at least two weeks post-natalizumab infusion, suggesting that trafficking...
of these cells had been interrupted. Less consistent findings were found for T cells expressing L-selectin. The results of the studies described in this chapter will be discussed further in section 9.5.2.
Chapter 8
Patterns of T-cell activation antigen expression in patients with inflammatory bowel disease.

8.1 Abstract

Aims
To compare patterns of expression of peripheral blood lymphocyte activation antigens in patients with newly-diagnosed and chronic inflammatory bowel disease. To examine the relationship between disease activity and activation antigen expression.

Methods
Peripheral blood samples were taken from patients with active IBD, including those whose disease was newly-diagnosed and untreated. Control samples were taken from healthy volunteers and patients with other causes of bowel inflammation. Lymphocytes were extracted and activation antigens were analysed by multi-colour FACS.

Results
Consistent correlations between NK-type cell and T cells expressing activation antigens were detected in IBD patients, to a lesser extent in disease controls, but not healthy volunteers. Proportions of peripheral blood lymphocytes expressing activation antigens were similar between all groups. Some small differences in NK-type cell proportions, TCRαβ+ and B cells were noted between IBD patients with new onset compared to those with chronic disease.

Conclusions
IBD patients exhibit consistent inter-relationships between NK-type cells and activated T cells which appear to be unrelated to disease activity. Some of these relationships also exist to a lesser extent in patients with non-IBD bowel inflammation, suggesting that these patterns may not be necessarily specific to IBD.
8.2 Introduction

Recognition that certain genetic manipulations in animals results in IBD-like conditions, has led to the hypothesis that IBD itself may be a manifestation of immune dysregulation. As described in 2.1.4 and 2.2, mice genetically deficient in the ‘anti-inflammatory’ cytokines, IL-2 or IL-10, develop an IBD-like condition, provided that they are not reared in a ‘germ-free’ environment {Sadlack, Merz, et al. 1993} {Kuhn, Lohler, et al. 1993}. Depleting mice of T-cell receptor alpha-beta (TCRαβ) cells has similar effects {Mombaerts, Mizoguchi, et al. 1993}. Furthermore, studies by Powrie et al. have shown that mice with severe-combined immunodeficiency develop IBD if their absent T-cells are replete with CD45RO+ cells, highlighting the importance of these cells in generating mucosal inflammation {Powrie, Carlino, et al. 1996}.

The results of multi-colour FACS of peripheral blood lymphocytes from some of the first patients to be screened for the natalizumab studies described in Chapters 4 and 5, raised the possibility that different patterns of immune dysregulation may occur in these patients, which are not necessarily related to disease activity, duration or immunosuppressant drug usage. Other workers have suggested this previously {Roman, Manzano, et al. 1996}.

The hypothesis for this study was that primary immune dysregulation occurs in IBD, which becomes masked by the effects of concomitant medication and disease chronicity. The aim, therefore, was to examine the relationship between IBD and lymphocyte activation, by comparing activation antigens and NK cell markers on peripheral blood lymphocytes from patients with newly-diagnosed, untreated disease with those with chronic IBD patients, healthy volunteers and patients with other causes of inflamed bowel mucosa.

8.3 Methods

8.3.1 Patients

IBD patients attending the departments of Medicine or Paediatric Gastroenterology at the Royal Free Hospital, Hampstead were selected for further study. The diagnosis of Crohn’s disease or ulcerative colitis needed to have been confirmed by radiology (Crohn’s disease patients only) or histology, in addition to
a typical history and/or examination findings of IBD [Riis 1990]. ‘Newly-diagnosed’, untreated patients were identified as those whose IBD confirmatory tests had been performed within the last three months and who had not commenced oral or rectal IBD medication, including 5-amino-salicylate drugs. ‘Inflamed’ disease control subjects were selected from consecutive patients who presented with microbiological and/or histological evidence of infectious enteritis, or histological evidence of ischaemic colitis or acute appendicitis. Organ-transplant recipients, patients with HIV infection or any other significant co-morbidity (atopy or autoimmune disease) and patients receiving cyclosporin, methotrexate or tacrolimus were excluded. Healthy volunteer-subjects were recruited from medical school staff, but those who had suffered any acute gastrointestinal illness during the preceding three months were excluded from further analysis. The study was approved by the Local Ethics Committee of the Royal Free NHS Trust Ethics Committee and written informed consent to participate was obtained from all patients or patients’ parents/guardians prior to study entry.

8.3.2 Clinical information
Details of age, sex, disease duration and ongoing medication were recorded for all patients (IBD and ‘inflamed’) in addition to completing a simple symptom questionnaire, from which both the Crohn’s disease activity index [Best, Becktel, et al. 1976] and Powell-Tuck ulcerative colitis activity scores [Powell-Tuck, Bown, et al. 1978] were calculated, regardless of the diagnosis at the time of presentation.

8.3.3 Peripheral blood samples
Venous blood was taken from all participants for measurement of full blood count, ESR, CRP and peripheral blood lymphocytes (PBLs). As far as possible, samples were taken from patients with newly-diagnosed disease prior to commencing any oral IBD medication, including 5-amino-salicylate drugs. Follow-up samples were taken from IBD patients at approximately three months after the initial sample, regardless of their disease activity state. Where possible, repeat samples were also taken once patients were in clinical remission, the time interval between initial and remission samples being recorded for each patient. Healthy
volunteers found to have elevated inflammatory markers or total white counts were excluded from further analysis, as these findings may have represented concurrent disease, such as upper respiratory tract infection. The Lymphoprep™ technique (Nycomed, Norway) was used to isolate PBLs from lithium-heparinised blood samples prior to analysis by multi-colour fluorescence-activated cell sorter (FACS; Becton Dickinson, Oxford, U.K.) in conjunction with Consort 30 software, as described and validated previously {Amlot, Tahami, et al. 1996}. The percentages of PBLs expressing the following markers were measured: CD19 (B cell), TCRαβ (T cell), CD3 (pan-T cell), TCRγδ (T cell), CD4 (helper/Th-1 T cell), CD8 (cytotoxic/suppressor T cell) and CD16 (NK cell). The percentages of TCRαβ cells expressing the activation antigens CD38, CD25 (α chain of the interleukin-2 receptor), CD26, CD69 and HLA-DR were measured, in addition to naïve (CD45RA) and memory (CD45RO) T cell subsets and CD57+CD3+ cells (NK-T cells). The percentage of cytotoxic/suppressor T cells (CD8+) expressing the activation antigens CD28 and HLA-DR were also measured. Sources of the antibodies used are listed in Appendix III.

8.3.4 Statistical analysis

Participants were categorised as follows for statistical analysis: healthy controls, inflamed controls, Crohn’s disease, ulcerative colitis and proctitis (i.e. histologically-confirmed rectal inflammation only). IBD patients were also subdivided into those with newly-diagnosed, untreated disease and those with chronic disease, defined as a time limit of three months since the date of diagnosis. Data from screening analyses of patients who were considered for the trials of natalizumab described in Chapters 4 and 5, were included in the chronic treated disease group. CRP was used as a marker of disease activity for patients in the disease control group. One-way analysis of variance (ANOVA) with a Bonferroni post-test was used to test for significant differences (p<0.05) between subject groups. Spearman’s tests were used to measure correlations between expression of different lymphocytes and clinical parameters. The following were combined to facilitate analyses of correlation due to small numbers in individual groups; proctitis and ulcerative colitis groups and newly diagnosed and chronic groups.
8.4 Results

8.4.1 Patients

84 IBD patients were selected, 47 of whom had Crohn’s disease, 28 had ulcerative colitis and nine had non-specific ulcerative proctitis. Samples were also taken from 18 healthy volunteers and from 11 patients with histological or microbiological evidence of infectious or ischaemic enteritis, who formed the ‘disease control’ group. The causes of their ‘disease’ were as follows: acute appendicitis two patients, Campylobacter infection two patients, Salmonella enteritidis infection two patients, Giardia infection one patient, ischaemic colitis one patient, and three patients with histological evidence of infectious colitis but pathogen unknown. Follow-up blood samples were taken from 47 (56%) IBD patients at three months and/or once patients were in remission. The mean age, disease duration, sex distribution and treatment profiles are shown in Tables 8.1 and 8.2. Overall, 26 of the IBD patients (31%) had newly-diagnosed, untreated disease at the time of initial blood sampling (11 Crohn’s disease, 14 ulcerative colitis and nine proctitis patients). Whilst patients’ age did not differ significantly between newly-diagnosed and ‘chronic’ Crohn’s disease patients, newly-diagnosed ulcerative colitis patients were significantly younger than patients with chronic disease (p=0.003). Both groups of new patients were significantly younger than healthy and disease controls (p<0.01 new IBD compared with either control group).

<table>
<thead>
<tr>
<th>Table 8.1 Patient demographics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Proctitis</td>
</tr>
<tr>
<td>Healthy controls</td>
</tr>
<tr>
<td>Disease controls</td>
</tr>
</tbody>
</table>

8.4.2 Disease activity and site

At the time of the initial blood sample, most IBD patients had active disease, as defined by a CDAI score of greater than 150 points (n=74; 88%) or Powell-Tuck score greater than 4 points (n=31; 84%). The mean CDAI score for Crohn’s
disease patients was 247 points (range 64-436) and the mean Powell-Tuck score was 8 points (range 1-12 points). Patients who had follow-up samples analysed at 12 weeks had significantly lower disease activity scores at this time-point compared to baseline values (Crohn’s disease p=0.04, ulcerative colitis p=0.002). There was no significant difference between baseline activity scores of patients who had follow-up at 12 weeks compared to those who did not. With respect to disease site, most Crohn’s disease patients (69%) had colonic involvement, the remainder (31%) having small bowel disease alone. Thirteen (28%) patients had colonic disease alone, whilst peri-anal disease had been experienced by 29% of Crohn’s disease patients. Thirteen (46%) ulcerative colitis patients had pancolitis and the remaining 15 patients had disease limited to the descending colon.

8.4.3 Concurrent treatment (Table 8.2)

Forty-four patients (52%) were on no immunosuppressant treatment at the time of the first blood sample, most of whom (34 patients) had newly-diagnosed disease, whilst 10 patients with chronic disease were on no treatment. Most remaining patients were taking oral mesalazine (34 patients: 40%), often in combination with corticosteroids, in the case of Crohn’s disease patients. A minority of IBD patients were receiving azathioprine (ten patients; 12%). The treatment profiles of patients at twelve weeks follow-up can be seen in Table 8.2. Treatments at twelve weeks follow-up differ, in that a significantly higher proportion of patients were receiving each of the three categories of IBD therapy (corticosteroids 66%, mesalazine 87%, azathioprine 32%).

<table>
<thead>
<tr>
<th>Oral Medication</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Proctitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks n=47</td>
<td>12 weeks n=31</td>
<td>0 weeks n=28</td>
</tr>
<tr>
<td>5-aminosalicylates</td>
<td>22 28</td>
<td>12 13</td>
<td>0</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>19 24</td>
<td>2 7</td>
<td>0</td>
</tr>
<tr>
<td>Azathioprine/ 6MP</td>
<td>8 12</td>
<td>2 3</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>0 3</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Nil</td>
<td>20 4</td>
<td>16 1</td>
<td>9 1</td>
</tr>
</tbody>
</table>
8.4.4 Basic lymphocyte subsets

The proportions of peripheral blood lymphocytes expressing TCRαβ, TCRγδ, CD19, CD4, CD8, CD45RO and CD45RA did not differ significantly between disease groups (Crohn’s disease, ulcerative colitis and proctitis) or between disease groups and controls (‘disease’ and healthy) at baseline (Table 8.3). Total lymphocyte counts and the ratio of CD4: CD8 lymphocyte ratios were also similar between groups.

8.4.5 Lymphocyte activation marker expression

The percentage of T cells expressing the following activation antigens did not differ significantly between any of the different groups; CD25, CD26, CD8CD28, CD8DR, CD69, CD38, CD57, HLA-DR (Table 8.4).

---

Table 8.3 Basic lymphocyte subsets

<table>
<thead>
<tr>
<th></th>
<th>Crohn’s Disease</th>
<th>Ulcerative colitis</th>
<th>Proctitis</th>
<th>Healthy Controls</th>
<th>Inflammatory Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRαβ+</td>
<td>68.6 17.3</td>
<td>70.6 10.7</td>
<td>62.7 12.1</td>
<td>64.2 9.1</td>
<td>57.5 11.2</td>
</tr>
<tr>
<td>CD4+</td>
<td>44.6 16.4</td>
<td>42.8 15.9</td>
<td>53.1 12.5</td>
<td>49.6 11.5</td>
<td>41.8 11.3</td>
</tr>
<tr>
<td>CD8+</td>
<td>25.8 8.1</td>
<td>31.1 11.5</td>
<td>53.1 12.5</td>
<td>21.1 6.1</td>
<td>25.7 8.4</td>
</tr>
<tr>
<td>CD4:8</td>
<td>2.0 1.2</td>
<td>1.8 1.4</td>
<td>2.6 0.7</td>
<td>2.5 1.2</td>
<td>2.1 1.9</td>
</tr>
<tr>
<td>CD19+</td>
<td>8.5 5.6</td>
<td>8.0 4.8</td>
<td>9.1 4.8</td>
<td>10.6 3.8</td>
<td>13.5 10.7</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>52.2 15.3</td>
<td>52.3 16.7</td>
<td>61.2 10.6</td>
<td>55.1 13.7</td>
<td>55.3 6.3</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>70.6 9.7</td>
<td>71.5 11.2</td>
<td>66.0 8.2</td>
<td>68.3 8.7</td>
<td>69.2 7.2</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>4.9 8.4</td>
<td>4.0 4.0</td>
<td>4.0 2.5</td>
<td>5.8 6.0</td>
<td>5.1 4.8</td>
</tr>
<tr>
<td>CD16+ (NK)</td>
<td>12.3 11.0</td>
<td>11.7 7.4</td>
<td>11.6 5.8</td>
<td>9.8 4.6</td>
<td>15.7 10.2</td>
</tr>
<tr>
<td>Lymphocyte Counts</td>
<td>1.5 0.9</td>
<td>1.9 0.9</td>
<td>1.7 0.4</td>
<td>2.3 0.4</td>
<td>1.4 0.4</td>
</tr>
</tbody>
</table>

Table 8.3 Numbers are means + SD. Subsets are expressed as percentage of total lymphocyte count, except for CD4:8 ratio.
Table 8.4  Lymphocyte activation antigen expression

<table>
<thead>
<tr>
<th></th>
<th>Crohn’s Disease</th>
<th>Ulcerative colitis</th>
<th>Proctitis</th>
<th>Healthy Controls</th>
<th>Inflammatory Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25+*</td>
<td>21.5 11.5</td>
<td>17.7 6.3</td>
<td>25.5 7.5</td>
<td>21.9 6.6</td>
<td>21.0 5.9</td>
</tr>
<tr>
<td>CD26+*</td>
<td>54.0 15.2</td>
<td>48.7 16.8</td>
<td>60.2 11.7</td>
<td>62.1 10.2</td>
<td>60.1 12.2</td>
</tr>
<tr>
<td>CD38+*</td>
<td>41.2 12.7</td>
<td>46.5 16.0</td>
<td>44.8 15.7</td>
<td>39.0 8.3</td>
<td>45.4 14.7</td>
</tr>
<tr>
<td>CD69+*</td>
<td>6.4  3.9</td>
<td>5.7  2.9</td>
<td>5.0  2.4</td>
<td>7.3  8.5</td>
<td>12.0 19.6</td>
</tr>
<tr>
<td>HLA-DR+*</td>
<td>21.7 15.4</td>
<td>26.1 14.3</td>
<td>24.4  9.5</td>
<td>16.3  8.5</td>
<td>28.6 13.2</td>
</tr>
<tr>
<td>CD8+DR+**</td>
<td>31.6 19.7</td>
<td>37.7 17.8</td>
<td>33.3 15.2</td>
<td>24.4 11.1</td>
<td>38.0 18.1</td>
</tr>
<tr>
<td>CD8+CD28+**</td>
<td>60.8 21.9</td>
<td>53.9 22.0</td>
<td>64.7 15.3</td>
<td>71.7 10.8</td>
<td>56.2 18.2</td>
</tr>
<tr>
<td>CD57+CD3+</td>
<td>9.9 10.0</td>
<td>11.2 10.5</td>
<td>6.0  5.7</td>
<td>5.2  3.6</td>
<td>7.9  7.6</td>
</tr>
</tbody>
</table>

Table 8.4  Numbers marked * are expressed as percentages of TCRαβ+ lymphocyte counts with SD in italics. Numbers marked ** are percentage of total CD8+ cells and remainder are percentage of total lymphocyte counts.

A sub-analysis comparing basic lymphocyte subsets of newly-diagnosed patients with those of patients with chronic IBD showed some differences. Firstly, in Crohn’s disease patients, NK cell proportions (CD16+CD3-) were significantly higher in patients with new, untreated disease compared to those whose disease was chronic (p=0.04) (Figure 8.1a). These differences persisted between the groups at the 12 week post-treatment sample (p=0.02)(Figure 8.2b), despite significant improvements in CDAI in each group (p=0.03 new; p=0.0006 chronic). Conversely, no significant differences in NK cell proportions were detected between patients with new and chronic disease initially, although a significant increase in NK cells occurred in new ulcerative colitis patients following 12 weeks of treatment (p=0.025) In ulcerative colitis patients, those with chronic disease had significantly higher TCRαβ proportions both before and at 12 weeks after treatment, compared with those with newly-diagnosed disease (p=0.0035 pre, 0.004 post)(Figures 8.2a and b). New ulcerative colitis patients had slightly lower CD4:CD8 ratios than those with chronic disease (p=0.04) but this difference did not persist between groups after 12 weeks of treatment. Ulcerative colitis patients with new-onset disease also had significantly higher B cell counts (p=0.03) compared to those with chronic disease prior to treatment (Figure 8.2a).
Figure 8.1 Comparison of lymphocyte subsets in patients with newly-diagnosed and chronic Crohn's disease.

Figure 8.1a Crohn's disease: week 0

Figure 8.1b Crohn's disease: week 12

Figure 8.1 (a&b) Comparison of T cell (TCRαβ+), B cell (CD19+) and NK cell (CD16+) proportions in patients with Crohn's disease. Significant differences are shown between new and chronic patients. Error bars are SD.

Figure 8.2 Comparison of lymphocyte subsets in patients with newly-diagnosed and chronic ulcerative colitis.

Figure 8.2a Ulcerative colitis week 0

Figure 8.2b Ulcerative colitis: week 12

Figure 8.2 (a&b) Comparison of T cell (TCRαβ+), B cell (CD19+) and NK cell (CD16+) proportions in patients with ulcerative colitis. Significant differences are shown between new and chronic patients. Error bars are SD.
8.4.6 Patterns of activation marker expression

A wide variation in the proportion of T cells expressing activation antigen was noted between patients within each disease group. Hence, patterns of lymphocyte activation antigen expression were examined using Spearman rank correlations. Because of small numbers, results of patients with newly-diagnosed disease and chronic disease were combined in a single disease group.

8.4.6.1 CD45RO+ cells

In Crohn’s disease patients, significant positive correlations were detected between CD45RO+ cells and T cells expressing CD69 (r=0.41), DR (r=0.58), CD8DR (r=0.47), and CD57 (r=0.47). CD45RO+ cells were also positively associated with age (r=0.35). NK cells were found to correlate positively with CD45RO+ cells in both Crohn’s disease patients and disease controls (r=0.34, r=0.71, respectively). Significant negative correlations were detected between CD45RO+ T cells and those expressing CD38 (r=-0.35), CD19 (r=-0.34) and CD8CD28 (r=-0.46) but this was also noted in the healthy control (CD38+, r=-0.82) and disease control groups (CD19+, r=-0.65; CD8+CD28+, r=-0.8). Similar patterns were seen in ulcerative colitis patients, with respect to T cells expressing DR, CD8DR, CD8CD28, NK cells, CD57 and age (see Table 8.5a).
Table 8.5a: Correlation between CD45RO+ T cells and other subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Disease controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRαβ+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>-0.3, 0.04</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD19+</td>
<td>-0.34, 0.02</td>
<td>NS</td>
<td>-0.65, 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>-0.32, 0.02</td>
<td>-0.61, &lt;0.0001</td>
<td>NS</td>
<td>-0.62, 0.01</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD16+</td>
<td>0.34, 0.02</td>
<td>0.44, 0.006</td>
<td>0.71, 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>CD25+</td>
<td>NS</td>
<td>0.39, 0.02</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD26+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD38+</td>
<td>-0.35, 0.02</td>
<td>NS</td>
<td>NS</td>
<td>-0.82, 0.0001</td>
</tr>
<tr>
<td>CD69+</td>
<td>0.41, 0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HLADR+</td>
<td>0.58, &lt;0.0001</td>
<td>0.61, &lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+/DR+</td>
<td>0.47, 0.001</td>
<td>0.52, 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+/CD28+</td>
<td>-0.46, 0.001</td>
<td>NS</td>
<td>-0.8, 0.005</td>
<td>NS</td>
</tr>
<tr>
<td>CD57+</td>
<td>0.47, 0.001</td>
<td>0.35, 0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age</td>
<td>0.35, 0.02</td>
<td>0.34, 0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Duration</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Activity</td>
<td>NS</td>
<td>-0.34, 0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

R=correlation coefficient for values where p<0.05.

● denotes significant positive correlation; ● denotes significant negative correlations

8.4.6.2 CD8+CD28+ cells

In both Crohn’s disease and ulcerative colitis groups, these cells correlated negatively with lymphocytes expressing DR (CD, UC; r=-0.79, r=-0.8), CD8DR (r=-0.78, r=-0.77), CD16 (r=-0.65, r=-0.37) and CD57CD3 (r=-0.72, r=-0.71). Similar correlations were detected between NK cells (CD16+) and CD8+CD28+ cells in disease controls (r=-0.81) but none of these findings were present in healthy controls. (see table 8.5b overleaf)
Table 8.5b: Correlation between CD8+CD28+ T cells and other subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Disease controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p</td>
<td>R</td>
<td>p</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>0.45</td>
<td>0.002</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>0.56</td>
<td>&lt;0.0001</td>
<td>0.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+</td>
<td>-0.38</td>
<td>0.009</td>
<td>-0.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4:8</td>
<td>0.59</td>
<td>&lt;0.0001</td>
<td>0.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD19+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.7</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>-0.45</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>-0.46</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD16+</td>
<td>-0.65</td>
<td>&lt;0.0001</td>
<td>-0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>CD25+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD26+</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>0.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD38+</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD69+</td>
<td>-0.37</td>
<td>0.02</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>-0.79</td>
<td>&lt;0.0001</td>
<td>-0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+DR+</td>
<td>-0.78</td>
<td>&lt;0.0001</td>
<td>-0.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD57+</td>
<td>-0.72</td>
<td>&lt;0.0001</td>
<td>-0.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Duration</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Activity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

R = correlation coefficient for values where p<0.05.

● denotes significant positive correlation; ■ denotes significant negative correlations

8.4.6.3 CD8+DR+ T cells

Generally, an inverse of the above (CD8+CD28+) correlations was found between CD8+DR+ cells and other lymphocyte marker expression. Once again, these finding were not present in either healthy or disease control groups (see Table 8.5c).
### Table 8.5c: Correlation between CD8+DR+ T cells and other subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Crohn's disease</th>
<th>Ulcerative colitis</th>
<th>Disease controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p</td>
<td>R</td>
<td>p</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>-0.31</td>
<td>0.03</td>
<td>-0.55</td>
<td>0.0005</td>
</tr>
<tr>
<td>CD8+</td>
<td>NS</td>
<td></td>
<td>0.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4:8</td>
<td>-0.37</td>
<td>0.01</td>
<td>-0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD19+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.47</td>
<td>0.001</td>
<td>0.52</td>
<td>0.001</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.35</td>
<td>0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD16+</td>
<td>0.39</td>
<td>0.007</td>
<td>0.38</td>
<td>0.02</td>
</tr>
<tr>
<td>CD25+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD26+</td>
<td>-0.42</td>
<td>0.003</td>
<td>-0.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD38+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD69+</td>
<td>0.47</td>
<td>0.002</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>DR+</td>
<td>0.86</td>
<td>&lt;0.0001</td>
<td>0.89</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+CD28+</td>
<td>-0.78</td>
<td>&lt;0.0001</td>
<td>-0.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD57+</td>
<td>0.63</td>
<td>&lt;0.0001</td>
<td>0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>0.39</td>
<td>0.008</td>
<td>0.46</td>
<td>0.005</td>
</tr>
<tr>
<td>Duration</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

R=correlation coefficient for values where p<0.05.

- ■ denotes significant positive correlation; □ denotes significant negative correlation.

#### 8.4.6.4 NK cells (CD16+) and NK T cells (CD3+CD57+)

The pattern of expression of these two markers paralleled each other in ulcerative colitis and Crohn’s disease groups, with occasional overlap with disease controls. Individual results are shown in Tables 8.5d and 8.5e below, but the pattern of NK marker expression followed that of CD8DR, namely positively associated with CD45RO and DR, but negatively associated with CD26, CD4, CD4:8 and CD28CD8. NK cells also correlated positively with γδ+ T cells (r=0.35) and negatively with αβ+ T cell proportions (r=−0.84) and disease activity (r=−0.34) and duration (r=−0.34) in Crohn’s disease patients only. In healthy controls correlations were noted between NK cells and CD25+ cells (r=0.56) only.
Table 8.5d: Correlation between CD57+ T cells and other subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Disease controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p</td>
<td>R</td>
<td>p</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>-0.4</td>
<td>0.006</td>
<td>-0.34</td>
<td>0.04</td>
</tr>
<tr>
<td>CD8+</td>
<td>0.44</td>
<td>0.002</td>
<td>0.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4:8</td>
<td>-0.49</td>
<td>0.0008</td>
<td>-0.56</td>
<td>0.0004</td>
</tr>
<tr>
<td>CD19+</td>
<td>-0.31</td>
<td>0.04</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.47</td>
<td>0.001</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD45RA+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.47</td>
<td>0.0009</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD16+</td>
<td>0.43</td>
<td>0.003</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD25+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD26+</td>
<td>-0.51</td>
<td>0.0003</td>
<td>-0.48</td>
<td>0.003</td>
</tr>
<tr>
<td>CD38+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD69+</td>
<td>0.41</td>
<td>0.01</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>DR+</td>
<td>0.69</td>
<td>&lt;0.0001</td>
<td>0.66</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+ DR+</td>
<td>0.63</td>
<td>&lt;0.0001</td>
<td>0.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD8+ CD28+</td>
<td>-0.72</td>
<td>&lt;0.0001</td>
<td>-0.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

R=correlation coefficient for values where p<0.05. ■ denotes significant positive correlation; □ denotes significant negative correlations

Table 8.5e: Correlation between NK cells (CD16+) and other subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Disease controls</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p</td>
<td>R</td>
<td>p</td>
</tr>
<tr>
<td>TCRαβ+</td>
<td>-0.84</td>
<td>&lt;0.0001</td>
<td>-0.59</td>
<td>0.0001</td>
</tr>
<tr>
<td>CD4+</td>
<td>-0.77</td>
<td>&lt;0.0001</td>
<td>-0.58</td>
<td>0.0002</td>
</tr>
<tr>
<td>CD8+</td>
<td>NS</td>
<td></td>
<td>0.34</td>
<td>0.04</td>
</tr>
<tr>
<td>CD4:8</td>
<td>-0.56</td>
<td>&lt;0.0001</td>
<td>-0.45</td>
<td>0.007</td>
</tr>
<tr>
<td>CD19+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.34</td>
<td>0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD45RA+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>0.35</td>
<td>0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD25+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD26+</td>
<td>0.36</td>
<td>0.01</td>
<td>-0.35</td>
<td>0.03</td>
</tr>
<tr>
<td>CD38+</td>
<td>NS</td>
<td></td>
<td>-0.36</td>
<td>0.03</td>
</tr>
<tr>
<td>CD69+</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>DR+</td>
<td>0.57</td>
<td>&lt;0.0001</td>
<td>0.35</td>
<td>0.03</td>
</tr>
<tr>
<td>CD8+ DR+</td>
<td>0.39</td>
<td>0.007</td>
<td>0.38</td>
<td>0.02</td>
</tr>
<tr>
<td>CD8+ CD28+</td>
<td>-0.65</td>
<td>&lt;0.0001</td>
<td>-0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>CD57+</td>
<td>0.43</td>
<td>0.003</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>-0.37</td>
<td>0.01</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>-0.34</td>
<td>0.02</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>
8.4.7 Unusual findings in individual patients

Two Crohn’s disease patients were noted to have extremely high proportions of circulating γδTCR+ cells (38% and 44% total lymphocyte count) accompanied by raised CRP (68g/l and 45g/l, respectively). These levels were approximately four, six and eight standard deviations greater than the mean γδTCR+ cell proportions in Crohn’s patients, healthy volunteers and disease controls, respectively. In the patient with the higher count, this proportion remained elevated at 48% at 12 weeks, having commenced azathioprine, although the disease remained active (CDAI 178 points; CRP 83g/l). The patient had disease affecting the terminal ileum and ascending colon, with previous perianal disease and mild orofacial involvement. An extended right hemicolectomy was performed 9 months after the initial sample was taken, following which, the patient experienced symptomatic remission. The initial samples were taken nine years from the time of diagnosis and in the absence of concurrent medication. Due to the unusual nature of this case, repeat FACS analysis was performed six weeks after surgery, which showed that the γδTCR+ count remained elevated at 37% despite having a normal CRP of 5g/l.

Secondly, three ulcerative colitis patients were observed to have very low CD4:CD8 ratios of not more than 0.4, compared to mean 1.8 and SD 1.4 for ulcerative colitis patients. All had relatively active disease with Powell-Tuck scores of 8, 11 and 12 points and disease affecting the left colon in one patient and total colitis in the remainder. All three patients had experienced symptoms for at least six years and one patient was on no treatment.

8.5 Conclusions

In this study, the predominant difference between IBD patients and control subjects was that of consistent relationships between different activated T cell subsets found in IBD patients only. Increased proportions of CD45-positive T cells, or T cells expressing CD25 were not detected in IBD patients compared to controls, in contrast with previous studies (see Chapter 9). Few differences in T cells expressing activation antigen were detected between patients with newly-diagnosed and chronic IBD,
although the groups were not well-matched in age. When newly-diagnosed and chronic IBD patient groups were combined for correlation analyses, IBD patients appeared to exhibit consistent relationships between some groups of T cell activation antigens. These relationships were found to a much lesser extent, or not at all in control groups. In particular, increased proportions of T cells expressing CD57 and CD8-HLADR were found to correlate with increased NK cells (CD16) and low proportions of CD8-positive T cells expressing CD28 in IBD patients. These findings suggest that a pattern of activation antigen expression may exist on circulating T cells in IBD, which is not found in healthy subjects or patients with other causes of gut inflammation. Finally, a small number of Crohn’s disease patients had extremely high circulating proportions of T cells expressing the gamma-delta T cell receptor. Such patients have been reported previously and are discussed further in the next chapter.

The results of these studies are discussed further in Chapter 9, section 9.6.
Chapter 9
Discussion

9.1 Natalizumab clinical studies

9.1.1 Safety and tolerability
Natalizumab was generally very well-tolerated, headache being the most commonly reported adverse event in both studies. Notably, headache frequency did not differ significantly from that of the placebo group in the Crohn’s disease study. Six patients in the natalizumab arm of the Crohn’s study and three patients in the ulcerative colitis study required admission during the 12-week follow-up period. Worsening underlying disease was the reason for admission in all six Crohn’s disease and one ulcerative colitis patient. Most of these patients had failed to respond to natalizumab initially, although three patients had severe disease relapse, having experienced a period of remission of up to five weeks following natalizumab. It was questioned whether the relapse appeared somewhat precipitous in two of these patients, having occurred over a period of 48 hours. It is possible that α4 blockade results in loss of regulation of leucocyte turnover, such that when natalizumab levels fall, there is an ‘overshoot’ in leucocyte recruitment to areas of inflammation. To date, none of the studies of α4 blockade in the colitic cotton-topped tamarin have commented on this possible phenomenon, although response-analyses are not quoted beyond a few days post-antibody infusion in any of the studies {Hesterberg, Winsor-Hines, et al. 1996}{Podolsky, Lobb, et al. 1993}. Long-term follow-up studies of infliximab in patients with IBD suggest that relapse occurs on drug withdrawal {Cohen, Tsang, et al. 2000}, but there is no evidence to suggest that the relapse which occurs is any more vigorous than would be expected. This area remains to be examined further in future studies of natalizumab.

9.1.2 Immunogenicity
As one would expect, natalizumab provoked a detectable antibody response in some patients, which was generally of short duration, i.e. limited to several weeks only. Antibodies were not anti-idiotype, but were neutralising in several patients. This latter
issue raises the possibility of loss of efficacy with repeated doses of natalizumab in certain patients. This phenomenon is well-described in studies of anti-TNFα antibodies {Hanauer 1999}. Given the small numbers of patients who developed antibodies of any significant duration, it is difficult to draw any firm conclusions regarding antibody production and its effect on clinical efficacy. Larger studies involving repeated doses of natalizumab will be instructive in this respect. No patients developed an anaphylactoid reaction to natalizumab, although this would have been an unexpected response to a single infusion in a study in which patients with prior exposure to mouse protein were excluded. The emergence of multiple monoclonal antibody-type therapies for IBD is likely to increase the likelihood of such reactions occurring.

9.1.3 Infection risk
It was anticipated that an increased risk of infection might occur in patients treated with natalizumab, since α4 integrin is widely expressed on all leucocytes except neutrophils, but this was not borne out by these studies. Interestingly, post-marketing surveillance of infliximab, has detected a higher than expected frequency of pulmonary tuberculosis infections in treatment recipients (Centocor web-page; infliximab product information). As mentioned in 9.1.1, one patient in the ulcerative colitis study developed Campylobacter jejuni enteritis, which precipitated a relapse of his colitis necessitating admission. It is impossible to know whether this infection was more severe than would otherwise have been expected if the patient had not received natalizumab. Interestingly, studies of a monoclonal antibody to α4β7 integrin in mice, have suggested that this integrin is important in CD8+ cell-mediated clearance of enteric rotavirus infection {Rose, Williams, et al. 1998}.

9.2 Efficacy of natalizumab in IBD
9.2.1 The impact of pharmacokinetics on efficacy
In both Crohn’s disease and ulcerative colitis patients, natalizumab resulted in statistically significant improvements in disease activity at two weeks compared to baseline, whilst no significant difference between natalizumab and placebo could be
demonstrated in the Crohn’s disease trial. In the Crohn’s disease study, however, the mean change in CDAI of 45 points at two weeks fell well short of the 103 points which had been used in the study’s power calculation. Natalizumab’s modest efficacy may have been partly due to suboptimal dosing. The half-life of natalizumab, namely 4.8 days in Crohn’s disease patients and 3.8 days in those with ulcerative colitis, was considerably shorter than the 8.7 days, predicted by healthy volunteer studies {Athena 1996}. It was also shorter than its half-life of 5.8 days in patients with multiple sclerosis {Sheremata, Vollmer, et al. 1999}. There are several possible explanations for this finding. First, the dynamics of α4 integrins in patients with chronic inflammatory conditions may differ from those of healthy volunteers. For example, it has been suggested that α4 integrins are more highly expressed on circulating and synovial leucocytes of patients with rheumatoid arthritis {Laffon, Garcia-Vicuna, et al. 1991} and IBD {Meenan, Spaans, et al. 1997}. Thus the concentration used may have been insufficient to cause adequate blockade of α4 integrin turnover, leading to suboptimal efficacy.

Alternatively, the receptor kinetics of α4 integrins may be altered in inflammatory conditions, such that the turnover at the cell surface is greater, leading to a greater concentration of antibody binding to each cell. Second, the half-life of leucocytes themselves may be shortened in such conditions, with many being lost into ‘sump’ sites such as stool and areas of chronic inflammation. For example, it is recognised that stool from IBD patients has higher leucocyte concentration than that of healthy control subjects {Handy, Ghosh, et al. 1995}. Serial radiolabelled white cell-scans of IBD patients have also demonstrated an increased flux of leucocytes into stool from the gut mucosa, emphasising the importance of this route of clearance of leucocyte-targeted monoclonal antibodies {Saverymuttu, Camillieri, et al. 1986}.

Another IgG4 monoclonal antibody, CDP571 (anti-TNFα antibody), has also been found to have a shorter half-life in Crohn’s disease patients than that predicted by healthy volunteer studies {Stack, Mann, et al. 1997}, suggesting that this might be a common problem for monoclonal antibody treatments in IBD. Lastly, natalizumab’s
efficacy duration may be shorter than other biologics because it does not fix complement and thus is unable to lyse the cells to which it binds. This contrasts with IgG1 complement-fixing antibodies such as infliximab (Scallon, Moore, et al. 1995). The efficacy of natalizumab therefore, may be more directly dependent on its plasma half-life.

Since these studies were completed, it has been found that a minimum serum natalizumab concentration of 5mcg/ml is required to produce blockade at least 80% of circulating membrane-bound α4 integrins (Elan Pharma Ltd., personal communication). Serum concentrations had fallen below this value in all patients by four weeks, which may help to explain natalizumab’s poorly sustained efficacy.

9.2.2 Patient characteristics and response

In patients with Crohn’s disease neither CDAI at study entry nor patients’ age correlated with their response, whilst patients with longstanding disease appeared to be less likely to respond to natalizumab at two weeks. Numbers of patients treated were too small to allow subgroup analyses of response to natalizumab according to disease distribution. Clinical factors that predict response to other biologics have been examined in some large multicentre studies. For example, some workers have suggested that pANCA positive patients may be less likely to respond to infliximab than those who have positive antibodies to Saccharomyces cerevisiae, who more often have isolated small bowel disease (Kam, Vasiliauskas, et al. 1999). This observation has been disputed by other groups (Vermeire, Joossens, et al. 2000). Genetic polymorphisms of the gene encoding the protein being targeted have also been suggested as response determinants (Vermeire, Joossens, et al. 2000), but human polymorphisms of α4 integrins have yet to be established.

Patients recruited to both studies were relatively heterogeneous with respect to prior medication. Seventy-five percent of Crohn’s patients and just 25% of ulcerative colitis patients were taking corticosteroids at study entry. Notably, some of these patients did not have truly recalcitrant disease, since they had not yet tried
azathioprine or other second line immunosuppressant agents. Patient numbers in each study were too small to permit feasible analysis of correlation between response to natalizumab and concomitant drugs at study entry. It would be interesting to determine in future studies, whether concomitant azathioprine treatment affects response to natalizumab, since this has been found to affect Crohn’s disease patients’ response to infliximab (Pittman, Toy, et al. 2000), suggesting some form of synergism between these two therapies.

9.2.3 Inflammatory markers and response
Although significant changes in CRP were seen in both Crohn’s disease and ulcerative colitis studies, they did not appear to predict response to natalizumab. This is perhaps understandable in ulcerative colitis patients, since changes in disease activity are not necessarily reflected in CRP or ESR results. Immunological factors and response to natalizumab will be discussed below in section 9.5.

9.3 Trial design learning points from Crohn’s disease studies
9.3.1 End-points
The end-point of trials of single dose biologics in IBD is commonly the comparison of response of randomised groups at four weeks post-treatment. In many cases, this time-point is likely to be derived from expectations of the new therapy’s duration of action, or from experience of corticosteroids, following which 60% of Crohn’s disease patients are likely to be in remission at four weeks (Rutgeerts, Lofberg, et al. 1994). Additionally, four weeks may be chosen for practical reasons, since it represents the maximum duration most patients can tolerate any lack of improvement, i.e. after this period most who have not responded will require rescue therapy, which may constitute withdrawal in some trials. This is borne out by the findings in the Crohn’s disease study, in which 75% of placebo-treated patients required rescue medication by four weeks (Figure 4.3). An end-point of two weeks was chosen in this study, however, because in vitro studies had suggested that a minimum serum concentration of 1 to 2 μg/ml of natalizumab was required to produce blockade of at least 80% of circulating α4 integrins. Healthy volunteer studies had suggested that
this serum concentration persisted to between three and four weeks after a single natalizumab infusion {Athena 1996}, thus a two week end-point was felt to be more appropriate than four. Further *in vitro* binding assays of natalizumab conducted after the start of these clinical studies have suggested that a serum concentration of at least 5μg/ml is required to produce adequate saturation of circulating α4 integrins. Since all patients had a serum concentration at four weeks which fell well short of this value, it is fortunate that a two week end-point was selected for this study.

9.3.2 CDAI limitations

The CDAI was selected for this study because it has been well-validated in previous IBD trials. It has inherent weaknesses as a score system, principally due to its largely subjective component and also because it requires a high degree of patient compliance. With respect to subjectivity, the factor in the score system with the highest weighting is ‘general well-being’ (weighting factor of x7). The range of possible scores in this category is thus from 49 to 196 points and a change in general well-being by one point can make a difference of seven points in the CDAI score, i.e. small changes in symptoms may alter the end CDAI substantially. Non-objective measures include the haematocrit value and percentage weight loss compared to standard weight for height. The Crohn’s disease natalizumab study illustrates how care must be taken in treating anaemia during such trials, since a blood transfusion can impact upon the CDAI so that the patient appears in remission. For example, patient number 27 (placebo group) received a blood transfusion after week two, following which the CDAI score fell by 45 points. Blood transfusion or iron therapy commenced after the study start should probably also be considered as rescue therapy in trials depending on the CDAI.

9.4 Learning points in trial design from ulcerative colitis study

9.4.1 Use of scoring systems in ulcerative colitis trials

The Powell-Tuck scoring system was selected for this study since it too has been well-validated and was felt to be a sufficiently objective measure of active disease. The basis of the score system has been explained in Chapter 3, but several limitations
of the method were encountered in this study. First, frequent sigmoidoscoposcopic examinations were not tolerated well by some patients, particularly those who had failed to respond to treatment or who had relapsed. Many of these patients declined examination at some point in the study, hence the sigmoidoscoposcopic part of the score was analysed separately. The advantage of rigid sigmoidoscopy is that it requires little equipment or preparation and is quick and easy to perform in the outpatient setting. However, the use of a fibre-optic instrument would probably have improved patient compliance with this aspect of the study.

Second, the Powell-Tuck score index is not a composite score of several days of diary cards, as the CDAI is, but assesses a patient's well-being on a single day. Thus the score system may falsely over- or under-estimate a patient's symptoms. Thirdly, the score system contains no objective measure of inflammation, other than the appearance of the rectum. Some trialists have tried to improve upon this by including measures of inflammation and haemoglobin in the score {Walmsley, Ayres, et al. 1998}.

9.4.2 Use of rectal medication
Interpreting change in disease activity in patients on rectal preparations may be difficult in clinical trials. In this study patients were asked to cease taking their rectal therapies at least two weeks before receiving natalizumab, so as to allow a period of stabilisation. The commencement of rectal preparations was then recorded as a rescue therapy. This method is perhaps less than ideal, since it may result in a deterioration in the patient's symptoms, particularly in patients with mostly distal colitis. Alternatively, the frequency of rectal preparation use could be used as an end-point. This method obviates the need to withdraw rectal preparations at the study start, which is likely to be more acceptable to patients. The disadvantage of this method is that rectal treatments used within the study need to be standardised in terms of dose and formulation, to enable accurate comparisons to be made between groups.
9.5 Immunological effects of natalizumab

9.5.1 Effects on basic leucocyte sub-types

9.5.1.1 Lymphocytes

A significant increase in circulating B and T lymphocytes occurred following natalizumab treatment in both Crohn’s disease and ulcerative colitis patients, which persisted to at least four weeks post-infusion in most patients, before returning to pre-treatment levels in all patients by eight weeks. This effect persisted for longer than would be predicted by mean serum natalizumab levels, since by four weeks, the serum level in all patients had fallen below $5\mu g/ml$, the value thought necessary to achieve blockade of at least 80% of circulating $\alpha 4$ integrins. Although these findings suggest that natalizumab interrupts lymphocyte trafficking, resulting in a rise in circulating counts, the change in lymphocyte counts did not correlate with reduction in CDAI or Powell-Tuck score (Chapter 6; Table 6c). This suggests that factors other than $\alpha 4$ integrin-mediated lymphocyte trafficking may potentiate disease activity in IBD. Alternatively, $\alpha 4$-mediated lymphocyte trafficking may have persisted sufficiently to produce continued disease activity, despite being partially inhibited by natalizumab.

9.5.1.2 Other leucocyte subsets

Significant elevation of eosinophils and monocytes were noted after natalizumab in both Crohn’s disease and ulcerative colitis patients (Chapter 6; Figures 6.1a and 6.1b). The increase in monocyte counts was less marked than that of eosinophils and no significant changes were detected in basophil or neutrophil counts. The latter result was in part expected, since neutrophils do not express $\alpha 4$ integrins. Basophils also express $\alpha 4$ integrins {Warner, Goldring, et al. 1995}, hence it was somewhat surprising that they did not appear to be affected by natalizumab. This may be related to the relative insensitivity of the technique used to measure this small cell sub-population, since basophil counts of less than $0.01 \times 10^9/l$ were registered as zero. Thus 30% of all basophil values were registered as undetectable.
9.5.1.3 Comparison with other studies

Increased levels of peripheral blood lymphocytes following natalizumab infusion were also detected in pre-clinical studies of cynomolgus monkeys and mice treated with natalizumab {Athena 1995}{Athena 1996}. Elevated lymphocyte levels were noted in studies of healthy volunteers {Athena 1996} and in patients with multiple sclerosis who received natalizumab in a randomised placebo-controlled trial {Tubridy, Behan, et al. 1999}. In each of these examples, it is proposed that natalizumab has disrupted lymphocyte trafficking to extravascular sites, resulting in an increase in the proportion of cells detectable in the intravascular compartment. Other investigators using antibodies which block different adhesion pathways have observed similar changes. For example, a rise in circulating leucocytes was noted in studies of α4β7 integrin monoclonal antibody in cotton-topped tamarins {Hesterberg, Winsor-Hines, et al. 1996}. Interestingly, leucocytosis also occurred in one of the earliest studies of α4 monoclonal antibodies in the cotton-topped tamarin, but by contrast, a predominant neutrophilia occurred {Podolsky, Lobb, et al. 1993}. A rise in circulating peripheral blood lymphocytes was also detected in studies of a different adhesion molecule interaction, namely ICAM-1 and β2 integrins (LFA-1 or CD11a/CD18). Firstly, trials of a monoclonal antibody to ICAM-1 in patients with rheumatoid arthritis resulted in elevated peripheral blood lymphocyte counts {Kavanaugh, Davis, et al. 1994}. Similarly, treatment of active Crohn’s disease with ICAM-1 antisense resulted in elevated circulating lymphocytes, particularly T cells which expressed β7 integrin {Bowen-Yacyshyn, Shanahan, et al. 1998}. Despite this observation, randomised placebo-controlled trials of ICAM-1 antisense have not shown it to be of significant clinical benefit in patients with active steroid-dependent Crohn’s disease {Yacyshyn, Chey, et al. 2000}.

9.5.2 Effects on adhesion molecule expression

9.5.2.1 α4 integrin and VCAM-1 interactions

One of the most striking immunological findings in these studies, was that a single infusion of natalizumab resulted in reduced serum soluble VCAM-1 concentrations in patients with ulcerative colitis and Crohn’s disease (Chapter 7; Figure 7.2). There are
several possible explanations for this finding, which has not been demonstrated in previous in natalizumab studies. Firstly, reduction in cell-cell interactions between α4+ leucocytes and VCAM-1-expressing endothelial cells may result in decreased shedding of VCAM-1 into the circulation. Secondly, the turnover of endothelial VCAM-1 expression may decrease, as a response to increased circulating intravascular leucocytes expressing α4 integrin; this would seem less likely. Finally, natalizumab may directly inhibit shedding of VCAM-1 from endothelial cell surfaces to the circulation. Overall, the results suggest that interruption of α4-mediated lymphocyte trafficking affects the turnover of endothelial VCAM-1 expression and subsequent release into the circulation.

It should be noted that the clinical significance of serum soluble VCAM-1 remains unknown, previous work having suggested that serum concentrations are unrelated to disease activity in IBD patients {Patel, Pall, et al. 1995}. Moreover, it can only be presumed that serum soluble VCAM-1 levels parallel expression on vascular endothelium, but the whether natalizumab directly affects mucosal expression of VCAM-1 and MAdCAM-1 remains unknown.

Minimal changes in circulating TCRαβ+CD49d+ cells were demonstrated following natalizumab infusion (Chapter 7; Figure 7.7). This was a somewhat unexpected result initially, since it was thought that the overall proportion of intravascular α4 integrin-expressing cells would increase following natalizumab infusion. There are several explanations for this discrepancy. Firstly, since this study was conducted, it is recognised that natalizumab directly reduces α4 integrin turnover on the T-cell surface (T.Yednock, personal communication), thus removing the linear relationship between FACS measurement of T cell α4 integrin expression and T cell numbers in the circulation. Second, the FACS method itself may have been unreliable, because natalizumab still bound to the surface of T cells may have interrupted the fluorescent-labelled CD49d FACS antibody. Finally, it is possible that natalizumab did not affect α4 integrin expression at all, but had a more general effect on lymphocytes.
9.5.2.2 ICAM-1 interactions

Previous studies have suggested that serum soluble ICAM-1 concentrations may be proportional to disease activity in IBD {Nielsen, Langholz, et al. 1994}, although others have disputed this finding {Goke, Hoffmann, et al. 1997}. Increased numbers of circulating CD54+ T cells post-infusion of natalizumab suggest that trafficking of these cells may be affected by blockade of α4 integrins. However, ICAM-1 is widely expressed on lymphocytes, thus the increase demonstrated may represent an epiphenomenon of the general increase in circulating lymphocytes, rather than a specific α4 integrin-mediated effect, i.e. cells which express α4 integrins may co-express ICAM-1 (CD54) and be affected by natalizumab. Alternatively, natalizumab may have disrupted a specific interaction between α4 integrins and ICAM-1.

9.5.2.3 Selectin pathways

The effects of natalizumab infusion were less consistent with regard to cells expressing L-selectin (CD62L+) and serum soluble E-selectin, although CD62L+ T cells were increased in both Crohn’s and ulcerative colitis patients at two weeks and soluble E-selectin concentration was increased at two weeks in ulcerative colitis patients only. Significant changes in these markers were not demonstrated following natalizumab. The interplay between α4 integrins and selectins is not clearly understood, although both are integral to leucocyte trafficking. L-selectin in conjunction with β7 integrins has been shown to be important in lymphocyte migration to Peyer’s patches {Kunkel, Ramos, et al. 1998}, whilst expression of P-selectin has been noted to be increased on vascular endothelium in inflamed areas of mucosa in IBD patients {Schurmann, Bishop, et al. 1995}.

9.5.3 Effects of natalizumab on other lymphocyte subsets and NK cells

9.5.3.1 NK cells

Studies of T cells expressing activation antigens revealed some consistent findings in both Crohn’s disease and ulcerative colitis patients. Firstly, NK cell counts were not affected by natalizumab infusion in either group. Similarly, NK-T cells (CD57+CD3+) were not affected in ulcerative colitis patients although were
significantly elevated at one week only in Crohn’s patients. In addition, NK cell counts had a significant inverse correlation with disease activity in Crohn’s disease patients.

These findings would suggest that NK-type cell trafficking is not affected by α4 blockade, raising the possibility that NK-type cells can use a different ligand pathway to migrate through vascular endothelium. This is contrary to studies that suggest that NK cell adhesion to VCAM-1 and fibronectin is mediated by α4 integrins {Gismondi, Morrone, et al. 1991}. Notably, however, a study of the effects of a different monoclonal antibody to α4 integrins, Act-1, showed that NK cell adhesion to VCAM-1 was not completely inhibited by α4 blockade {Perez-Villar, Zapata, et al. 1996}. These findings may be relevant to the efficacy of natalizumab in IBD, since NK cells may be important in generating inflammation as discussed in Section 2.1.2.

9.5.3.2 Gamma-delta T cells
Another cell subset which did not appear to be affected by α4 blockade was that of T cells expressing the γδ T cell receptor (TCRγδ+ cells). The role of these cells in IBD remains unclear, given that there are reports suggesting that they are involved in both generating and preventing inflammation (Section 2.1.3). The lack of effect of α4 integrin blockade on TCRγδ+ cells might support the latter argument.

9.5.3.3 Activated T cells
Significant increases in counts of T cells expressing the activation markers CD25, CD26, CD45RA, CD45RO, HLA-DR, CD8DR and CD8CD28 occurred in both groups of IBD patients until at least the first week post-infusion. These results suggest that T cells which express α4 integrins are also likely to co-express these markers of activation. The inconsistencies between Crohn’s disease and ulcerative colitis patients of the early activation markers CD38+ and CD69+ are not easy to explain. Missing data for these markers in the Crohn’s group was equivalent to that of other markers (8.3%) and similar to that of the ulcerative colitis group (10%). Numbers of patients in the ulcerative colitis groups were small however, (n=10) and larger studies might
help to establish whether $\alpha_4$ integrin blockade truly exerts different effects on these cell populations in Crohn's disease and ulcerative colitis patients.

Finally, some unexpected changes in T cells expressing activation antigens were found in the placebo group. Changes which occurred beyond week two are harder to evaluate, since the effects of rescue corticosteroids may have been important factors in a third of patients (n=4) who commenced these drugs after this time-point. Most changes however, represented a decrease in activated T cells compared to baseline values at one week post-placebo (TCR$\alpha$-$\beta$+ cells expressing CD45RO, CD38 and HLA-DR and total CD8+ counts). Complete data-sets were available for all 11 patients analysed at this time-point, thus the data may reflect the changes in activated T cells which occur with untreated active disease in some Crohn's disease patients. The results need to be interpreted with caution however, since only small numbers of patients were studied and further study of a larger placebo group would help to substantiate the findings.

9.6 FACS analysis of peripheral blood lymphocytes of newly-diagnosed versus chronic IBD patients

9.6.1 Comparison between IBD patients and controls

This study identified patterns of peripheral blood lymphocyte activation in IBD, which were not entirely consistent with findings of previous workers. For example, increased expression of HLA-DR, either on lymphocytes generally or on CD8+ cells, has been noted in Crohn's disease patients {van Tol, Verspaget, et al. 1992} {Selby, Janossy, et al. 1983}. Furthermore, decreased CD25+ T cell proportions and an inverse relationship between disease activity and peripheral NK cell proportions has been demonstrated in Crohn's disease patients by other workers {Kontiainen, Scheinin, et al. 1996}. This study also differs from some previous studies, in that it did not identify upregulation of T cell IL-2 receptor (CD25) or CD45RO+ on peripheral T cells in Crohn's disease patients {Senju, Hulstaert, et al. 1991} {Roman, Manzano, et al. 1996}. There is no obvious explanation for these differences,
although differences between studies of FACS gating mechanisms, FACS antibody specificity or patient population (UK versus Spanish) may account for this.

9.6.2 Comparison between patients with newly-diagnosed and chronic IBD

Significant differences between patients with long-standing and newly-diagnosed disease were detected in a small number of lymphocyte subsets only. The patterns of lymphocyte activation identified in this study also appeared to be unrelated to concurrent therapy, although the three-month interval between pre-treatment and post-treatment samples may have been too short a time in which to expect a treatment-related change. Additionally, the groups were heterogenous in terms of the drugs received, and changes effected by a single drug (e.g. azathioprine) may have occurred in too small a number of patients to be detected significantly by this study.

In addition, the groups were not well-matched in age, due to the proportion of paediatric new patients. Whilst patients' age did not differ significantly between 'new' and 'chronic' Crohn's disease patients, 'new' ulcerative colitis patients were significantly younger than 'chronic' patients. Both groups of new patients were significantly younger than healthy and disease controls. Lack of paediatric controls and paediatric patients with 'chronic' IBD is a probable flaw in the design of this study which may have affected the results of subgroup analyses. In particular, lower TCRαβ+ cells in ulcerative colitis patients may be age-related, since some workers report there to be a steady increase in T cell counts throughout childhood {Lin, Chou, et al. 1998}. Conversely, others have suggested that lymphocyte subsets in children are equivalent to those of adults by age 13 {Melaranci, Ciaffi, et al. 1992}. In this study, two of the 13 (15%) new Crohn's patients and five of the 25 (20%) new ulcerative colitis patients were under 13 years of age.
9.6.3 Correlation between different lymphocyte activation markers

In addition to looking at individual subsets, multicolour FACS permitted analysis of patterns of expression of co-existent T cell activation antigens. These analyses suggested that common patterns of expression of these antigens exist in IBD which appear unrelated to disease activity or concurrent medication and are not found in healthy controls. For the purpose of these studies, ‘new’ and ‘chronic’ IBD patients were grouped together for comparison with controls. The dominant pattern was that of strong positive correlation between expression of HLA-DR on CD8+ cells, CD57 on CD3+ cells and CD16+ cells (NK cells) in association with a negative correlation with expression of CD28 on CD8+ cells in both Crohn’s disease and ulcerative colitis patients. Some of these correlations were found in disease controls but only the correlation between CD8DR and CD57 on T cells was present in healthy controls. The significance of these findings is unclear. They may represent reciprocal changes which occur at the lymphocyte surface in response to gut inflammation. It is unclear whether some of the positive correlations between CD markers identified in this study represent an ‘activated T cell phenotype’ which may develop in IBD. For example, the positive correlations found on TCRαβ+ cells between CD45RO, CD69, HLA-DR, CD57 and CD8DR may represent the pattern of co-expression of these markers when T cells become activated. The correlations found between NK cells (CD16+) and other T cell subsets are unlikely to represent co-expression however, since these cells were specifically CD3 negative (i.e. non-T cell). Repeating the studies using more than three markers per cell (e.g. CD45RO+HLA-DR+CD69+CD57+) might help to determine this. In addition, including samples from a non-enteric inflammatory control population might have helped to establish whether the findings were specific to gastro-intestinal causes of inflammation.

9.6.4 Individual patient anomalies

Finally, a small number of patients in these studies exhibited individual lymphocyte subset proportions which differed widely from those of the remainder of the group. Firstly, one Crohn’s disease patient had persistently high proportions of γδTCR+ cells (>40% total lymphocyte count) which remained at this level despite resection of the
affected terminal ileum and ascending colon. Reports of similar cases exist in the literature \cite{Soderstrom1996,Giacomelli1994}, but the significance of this observation is unclear. Consistent with the conclusions of the former group, this patient’s $\gamma\delta$TCR+ level appeared unrelated to disease activity.

Secondly, a small group of ulcerative colitis patients had particularly low CD4:CD8 cell ratios, a phenomenon which has also been noted in patients with Crohn’s disease, suggesting upregulation of CD8+ and/or down regulation of CD4+ cells in these patients \cite{Henry1994,Neil1994}. Other workers have questioned these findings \cite{Selby1983,Pallone1985}.
Chapter 10
Conclusions and plans for future work

10.1 Conclusions

Natalizumab and inflammatory bowel disease
These studies suggest that blockade of α4 integrins by natalizumab may be of clinical benefit in active Crohn’s disease and ulcerative colitis, although the dose used may have been suboptimal. Larger studies are required to establish its clinical properties, including a randomised double-blind placebo-controlled study in ulcerative colitis patients. Natalizumab appears to be relatively safe, although the effects of repeated doses remain unknown.

Natalizumab and lymphocyte trafficking
A single infusion of natalizumab results in elevated counts of peripheral blood leucocyte counts for at least four weeks, B and T cells and eosinophils being particularly affected. Cells expressing ICAM-1 and L-selectin were also affected, albeit to a lesser degree. These findings suggest that natalizumab interrupts leucocyte trafficking.

α4 blockade by natalizumab reduces serum soluble adhesion molecule levels.
Natalizumab reduces serum concentrations of soluble VCAM-1 in patients with IBD. Elevated levels of E-selectin were also present post-infusion in ulcerative colitis patients. ICAM-1 levels were not affected in either group. These findings suggest that natalizumab disrupts VCAM-1 turnover.

Natalizumab affects the expression of activation antigens by lymphocytes.
Natalizumab infusion resulted in increased numbers of circulating T cells expressing the following markers of activity; CD45RO, CD45RO, CD25, CD26, HLA-DR, CD8CD28, CD8DR. Gamma-delta T cells and NK cells (CD16) were not affected by
natalizumab and NK-T cells (CD57) were elevated at one week in Crohn’s disease patients only.

**Lymphocyte profiles in newly-diagnosed and chronic IBD patients**

Patients with inflammatory bowel disease exhibited some consistent patterns of activation antigen expression, which were found to a lesser extent in disease controls but not healthy volunteers. NK cell proportions were greater in patients with newly-diagnosed, untreated Crohn’s disease compared to those with chronic disease. There was no evidence of primary immune dysfunction in patients with newly-diagnosed, untreated IBD.

10.2 Future Studies

10.2.1 Clinical studies of natalizumab in IBD patients

The trend to positive efficacy of natalizumab in patients with active Crohn’s disease described in Chapter 4, has led to the development of a multicentre dose-ranging study of natalizumab in active Crohn’s disease. I was one of the lead investigators involved in this study’s design, together with my supervisor, Professor Roy Pounder, and Elan Pharma Ltd.

Two hundred and forty patients with moderately active Crohn’s disease (CDAI>220 points) received one of the following treatments after 1:1:1:1 double-blind randomisation: placebo infusion repeated at one month, 3mg/kg infusion repeated at one month, 3mg/kg infusion with placebo at one month, 6mg/kg infusion repeated at one month. The study confirmed the efficacy of both 3mg/kg and 6mg/kg natalizumab doses when repeated at a one month interval in moderately active Crohn’s disease. The results were reported at the American Gastroenterological Association annual Digestive Diseases Week meeting in Atlanta in May 2001 {Antegren Publication Committee 2001}. Further studies will examine the efficacy of natalizumab in maintaining remission in active Crohn’s disease patients.
Together with Elan Pharma Ltd., I have also been involved in designing a randomised double-blind placebo-controlled study of natalizumab in patients with ulcerative colitis, which is due to commence in November 2001.

In view of the safety concerns of treating patients with repeated doses of murine-based monoclonal antibody preparations, developing a different form of \( \alpha_4 \) integrin inhibitor would be desirable, such as antisense oligonucleotides to inhibit \( \alpha_4 \) integrin expression. Safety analyses in future clinical studies will include assessment of long term outcome and measurement of evidence of drug-induced lupus syndrome, following the results of studies of infliximab in IBD patients {Hanauer 1999}.

10.2.2 Immunological effects of natalizumab

Natalizumab was found to reduce VCAM-1 levels for at least two weeks post-infusion. The significance of this effect in relation to its clinical efficacy is unclear. Since there is no significant difference in circulating VCAM-1 between patients with active IBD and healthy volunteers, one might expect that natalizumab would lower VCAM-1 in healthy subjects too. Analysis of VCAM-1 levels in serum taken from healthy volunteers who participated in Phase I studies of natalizumab would help to confirm this. Such studies may not be possible now, however, since VCAM-1 ELISAs would be unlikely give reliable results for these six year-old samples. Animal studies may also help to establish the effects of natalizumab on serum soluble VCAM-1.

In addition to studying the effects of natalizumab on serum soluble adhesion molecules, studies of colonic tissue would help to establish how natalizumab affects IBD pathogenesis. It would be interesting to discover whether a single natalizumab infusion reduces endothelial expression of VCAM-1 in enteric mucosa in addition to its effects on the circulating soluble form. Secondly, examining how natalizumab affects leucocyte subsets in colonic biopsies might illustrate how trafficking is disrupted after infusion. Alternatively, performing abdominal immunoscintigraphy of IBD patients treated with radiolabelled natalizumab could help to confirm whether
natalizumab interacts directly with leucocytes trafficking to the gut. Such studies have been performed with radiolabelled antibodies to E-selectin (Bhatti, Chapman, et al. 1998).

Finally, natalizumab raises levels of most circulating leucocyte subsets. No unexpectedly serious infections have developed in the patients exposed to date, but it may be helpful to study the effects of natalizumab on leucocyte function further. This could be performed *in vitro* using lymphocytes extracted from treated patients, or by studying the effects of natalizumab in animals. Why some leucocyte subsets did not seem to be affected by natalizumab (NK cells and gamma-delta T cells) may also deserve further investigation.

### 10.2.3 Patterns of activation antigen expression in IBD patients

The studies described in Chapter 8, which examined lymphocyte activation antigen expression in newly-diagnosed compared to chronic IBD patients, may have had somewhat ambitious or unrealistic aims. Conducting this type of study in a single centre demonstrated the difficulty of recruiting homogeneous patient groups, in terms of disease phenotype, age and duration, within a relatively short time.

In order to study the effects of treatments such as corticosteroids or 5-ASA drugs on T cell activation antigens, it might be better to design a prospective study based on the effects of individual therapies. Such studies could yield further information about the immune ‘phenotype’ of patients who do not respond to specific therapies, such as corticosteroids (Hearing, Norman, et al. 1999). With respect to studying how activation antigen expression varies with disease activity, it would be better to conduct the study prospectively over a much longer period e.g. two to three years. This might require rather smaller numbers of patients, since they would act as their own controls, and provide information on how lymphocyte activation antigen expression patterns vary through periods of activity and remission.
It is not known from the studies described in Chapter 8, whether the patterns of activation antigen expression found are unique to IBD patients. Similar patterns were found in inflammatory controls, but perhaps larger numbers of such control subjects are needed to confirm this, in addition to studying lymphocyte patterns in patients with other chronic inflammatory conditions such as rheumatoid arthritis or chronic vasculitis. It may be better to concentrate further studies on those lymphocyte subsets whose interrelationship patterns in IBD were most consistent, such as CD8+ cells expressing CD28 or HLA-DR, and T cells expressing CD57 or HLA-DR.
Appendix I
Sample Crohn’s disease activity index (CDAI) score sheet
{Best, Becktel, et al. 1976}* 

<table>
<thead>
<tr>
<th>Disease factor</th>
<th>Weighting factor</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of diarrhoeal stools during preceding week</td>
<td>x2</td>
<td></td>
</tr>
<tr>
<td>General well-being (sum of daily scores for week)</td>
<td>x7</td>
<td></td>
</tr>
<tr>
<td>0 = well, 1 = below par, 2 = poor, 3 = very poor, 4 = terrible.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain (sum of daily scores for week)</td>
<td>x4</td>
<td></td>
</tr>
<tr>
<td>0 = none, 1 = mild, 2 = moderate, 3 = severe.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-diarrhoeal medication</td>
<td>x30</td>
<td></td>
</tr>
<tr>
<td>0 = not taken, 1 = taken.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of extra-intestinal manifestations</td>
<td>x20</td>
<td></td>
</tr>
<tr>
<td>Iritis/ uveitis, arthralgia/ arthritis, skin or mouth lesions, new anorectal lesion, other fistulae, temp &gt;38°. (one point each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaemia</td>
<td>x6</td>
<td></td>
</tr>
<tr>
<td>Standard haematocrit– patient’s value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(47% male; 42% female)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage weight loss</td>
<td>x100</td>
<td></td>
</tr>
<tr>
<td>Standard weight** – patient’s weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal mass</td>
<td>x10</td>
<td></td>
</tr>
<tr>
<td>0 = absent, 2 = questionable, 5 = definite.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>&lt;150 points: remission; 150 - 400 points: mild to moderate; &gt;400 points: severe.</td>
</tr>
</tbody>
</table>

*Used in conjunction with weekly symptom diary card.

** Standard weight for build and sex according to Metropolitan Life tables.
Appendix II

Sample Powell-Tuck colitis activity index score sheet

{Powell-Tuck, Bown, et al. 1978}

<table>
<thead>
<tr>
<th>Disease factor</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>General health</td>
<td></td>
</tr>
<tr>
<td>0 = good, 1 = slightly impaired, 2 = activities reduced, 3 = unable to work.</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td></td>
</tr>
<tr>
<td>0 = absent, 1 = with bowel actions only, 2 = prolonged episodes.</td>
<td></td>
</tr>
<tr>
<td>Number of bowel motions daily</td>
<td></td>
</tr>
<tr>
<td>0 = &lt;3, 1 = 3-6, 2 = &gt;6.</td>
<td></td>
</tr>
<tr>
<td>Stool consistency</td>
<td></td>
</tr>
<tr>
<td>0 = formed, 1 = semi-formed, 2 = liquid.</td>
<td></td>
</tr>
<tr>
<td>Blood in stools</td>
<td></td>
</tr>
<tr>
<td>0 = absent, 1 = trace, 2 = frank.</td>
<td></td>
</tr>
<tr>
<td>Extra-intestinal manifestations</td>
<td></td>
</tr>
<tr>
<td>Iritis/ uveitis, arthralgia/ arthritis, erythema nodosum or pyoderma gangrenosum, mouth ulcers.</td>
<td></td>
</tr>
<tr>
<td>(1 point each for mild, 2 points for severe)</td>
<td></td>
</tr>
<tr>
<td>Abdominal tenderness</td>
<td></td>
</tr>
<tr>
<td>0 = absent, 1 = mild, 2 = marked, 3 = rebound.</td>
<td></td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td></td>
</tr>
<tr>
<td>0 = no, 1 = yes.</td>
<td></td>
</tr>
<tr>
<td>Appetite</td>
<td></td>
</tr>
<tr>
<td>0 = normal, 1 = reduced</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
</tr>
<tr>
<td>0 = absent, 1 = 37.1-38°C, &gt;38°C.</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix III

**Monoclonal antibodies used for FACS analysis**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>OKT3</td>
<td>Royal Free &amp; University College Medical School (RFUCMS), London, UK.</td>
</tr>
<tr>
<td>CD19</td>
<td>RFB9</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>TCRαβ</td>
<td>T10B9</td>
<td>Gift of S. Brown, VA Research Department, Lexington, Virginia, USA.</td>
</tr>
<tr>
<td>TCRγδ</td>
<td>TCR81</td>
<td>Perbio Science UK Limited, Tattenhall, UK.</td>
</tr>
<tr>
<td>CD4</td>
<td>RFT4</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD8</td>
<td>RFT8u</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD25</td>
<td>RFT5g2a</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD26</td>
<td>TA1</td>
<td>Beckman Coulter Ltd., High Wycombe, UK.</td>
</tr>
<tr>
<td>CD28</td>
<td>9.3</td>
<td>Gift of M Glennie, Southampton, University, UK.</td>
</tr>
<tr>
<td>CD38</td>
<td>RFT10</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD69</td>
<td>AIM</td>
<td>Beckman Coulter Ltd., High Wycombe, UK.</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>RFDR2</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD45RA</td>
<td>SN130</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD45RO</td>
<td>UCHL1</td>
<td>Gift of Peter Beverley, UCL, London, UK.</td>
</tr>
<tr>
<td>CD57</td>
<td>HNK1</td>
<td>Becton Dickinson, Oxford, UK.</td>
</tr>
<tr>
<td>CD7</td>
<td>RFT2</td>
<td>RFUCMS, London, UK.</td>
</tr>
<tr>
<td>CD16</td>
<td>Leu11b</td>
<td>Gift of S. Brown, VA Research Department, Lexington, Virginia, USA.</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>Beckman Coulter Ltd., High Wycombe, UK.</td>
</tr>
<tr>
<td>CD49d</td>
<td>VLA-4</td>
<td>Biosource International, Camarillo, California, USA.</td>
</tr>
<tr>
<td>Kappa light chain</td>
<td></td>
<td>Dako Ltd., Ely, Cambridgeshire, UK.</td>
</tr>
</tbody>
</table>
Appendix III continued
Suppliers of other reagents

**Natalizumab (Antegren™) and placebo:**
Elan Pharmaceuticals Europe Limited,
Abel Smith House,
Gunnels Wood Road,
Stevenage,
Hertfordshire SG1 2FG, UK.

**ELISA kits for serum adhesion molecule analyses:**
R&D Systems Europe Limited,
4-10 The Quadrant,
Barton Lane,
Abingdon,
Oxon OX14 3YS, UK.

**FACS analyser:**
Becton Dickinson UK Limited,
Between Towns Road,
Cowley,
Oxford OX4 3LY, UK.

**Lymphoprep™ reagents:**
Nycomed Pharma AS,
Langebjerg 1,
P.O. Box 88,
DK-4000 Roskilde, Denmark.
Appendix III continued
Suppliers of other reagents

FACS antibody suppliers:
Perbio Science UK Limited,
Century House,
High Street,
Tattenhall,
Cheshire CH3 9RJ, UK.

Beckman Coulter UK Limited,
Oakley Court,
Kingsmead Business Park,
London Road,
High Wycombe,
Buckinghamshire HP11 1JU, UK.

Biosource International,
542 Flynn Road,
Camarillo,
California,
USA 93012.

DAKO Limited,
Denmark House,
Angel Drove,
Ely,
Cambridgeshire CB7 4ET, UK.
Appendix IV

Precision of serum adhesion molecule ELISAs

Intra-assay analyses

<table>
<thead>
<tr>
<th>Plate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM A</td>
<td>325</td>
<td>355</td>
<td>318</td>
<td>110</td>
<td>237</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>327</td>
<td>375</td>
<td>338</td>
<td>107</td>
<td>241</td>
<td>114</td>
</tr>
<tr>
<td>Mean</td>
<td>326</td>
<td>365</td>
<td>328</td>
<td>108</td>
<td>239</td>
<td>120</td>
</tr>
<tr>
<td>SD</td>
<td>1</td>
<td>14</td>
<td>14</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>CV</td>
<td>0.3%</td>
<td>3.9%</td>
<td>4.2%</td>
<td>2.0%</td>
<td>1.2%</td>
<td>7%</td>
</tr>
<tr>
<td>VCAM B</td>
<td>129</td>
<td>349</td>
<td>170</td>
<td>177</td>
<td>236</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>365</td>
<td>172</td>
<td>188</td>
<td>265</td>
<td>100</td>
</tr>
<tr>
<td>Mean</td>
<td>127</td>
<td>357</td>
<td>171</td>
<td>183</td>
<td>250</td>
<td>95</td>
</tr>
<tr>
<td>SD</td>
<td>3</td>
<td>11</td>
<td>2</td>
<td>8</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>CV</td>
<td>2.7%</td>
<td>3.2%</td>
<td>1.0%</td>
<td>4.5%</td>
<td>8.1%</td>
<td>7.3%</td>
</tr>
<tr>
<td>ICAM 1 A</td>
<td>220</td>
<td>219</td>
<td>209</td>
<td>257</td>
<td>192</td>
<td>267</td>
</tr>
<tr>
<td></td>
<td>219</td>
<td>216</td>
<td>207</td>
<td>248</td>
<td>184</td>
<td>256</td>
</tr>
<tr>
<td>Mean</td>
<td>220</td>
<td>217</td>
<td>208</td>
<td>252</td>
<td>188</td>
<td>261</td>
</tr>
<tr>
<td>SD</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>CV</td>
<td>0.2%</td>
<td>1.1%</td>
<td>0.6%</td>
<td>2.7%</td>
<td>3.0%</td>
<td>2.8%</td>
</tr>
<tr>
<td>ICAM 1 B</td>
<td>172</td>
<td>117</td>
<td>106</td>
<td>141</td>
<td>100</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>172</td>
<td>136</td>
<td>89</td>
<td>124</td>
<td>103</td>
<td>138</td>
</tr>
<tr>
<td>Mean</td>
<td>172</td>
<td>127</td>
<td>97</td>
<td>133</td>
<td>102</td>
<td>141</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>CV</td>
<td>0%</td>
<td>10.5%</td>
<td>11.9%</td>
<td>9.3%</td>
<td>2.6%</td>
<td>2.7%</td>
</tr>
<tr>
<td>E-selectin A</td>
<td>66</td>
<td>53</td>
<td>42</td>
<td>46</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>58</td>
<td>40</td>
<td>46</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>67</td>
<td>56</td>
<td>41</td>
<td>46</td>
<td>44</td>
<td>13</td>
</tr>
<tr>
<td>SD</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CV</td>
<td>2.3%</td>
<td>6.4%</td>
<td>2.2%</td>
<td>0.9%</td>
<td>2.1%</td>
<td>7.0%</td>
</tr>
<tr>
<td>E-selectin B</td>
<td>45</td>
<td>47</td>
<td>50</td>
<td>43</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>48</td>
<td>49</td>
<td>44</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>Mean</td>
<td>47</td>
<td>48</td>
<td>50</td>
<td>44</td>
<td>39</td>
<td>30</td>
</tr>
<tr>
<td>SD</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CV</td>
<td>4.8%</td>
<td>2.6%</td>
<td>2.4%</td>
<td>2.4%</td>
<td>3.1%</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

Inter-assay precision

<table>
<thead>
<tr>
<th>Range of valid concentrations ng/ml</th>
<th>Concentration Plate A ng/ml</th>
<th>Concentration Plate B ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM</td>
<td>1326 - 2030</td>
<td>1839</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>241 - 342</td>
<td>263</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>36.6 - 80.1</td>
<td>63.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.3</td>
</tr>
</tbody>
</table>
Appendix V

Copies of Ethics Committee correspondence for Chapters 4, 5 and 8.
Dr Fiona Gordon  
Department of Academic Medicine

Dear Dr Gordon  

Re: 169-96

TREATMENT OF CROHN’S DISEASE WITH ANTI-INTEGRIN CHIMERIC MONOCLONAL ANTIBODY (ANTEGREN)

I am pleased to be able to inform you that your recent submission to the Ethical Practices Sub-Committee has now received approval by the majority of members, with no outstanding adverse comments, and is thus approved.

This approval will be formally documented at the next meeting of the full committee and meanwhile you are free to go ahead with your project.

Yours sincerely

Maureen Carroll  
Secretary  
Ethical Practices Sub-Committee

cc Mr J Farrell, Head of Pharmaceutical Services
Dr Fiona Gordon  
Department of Academic Medicine

Dear Dr Gordon

Re: 170-96

TREATMENT OF ACTIVE ULCERATIVE COLITIS WITH ANTI-INTEGRIN CHIMERIC MONOCLONAL ANTIBODY (ANTEGREN)

I am pleased to be able to inform you that your recent submission to the Ethical Practices Sub-Committee has now received approval by the majority of members, with no outstanding adverse comments, and is thus approved.

This approval will be formally documented at the next meeting of the full committee and meanwhile you are free to go ahead with your project.

Yours sincerely

Maureen Carroll  
Secretary  
Ethical Practices Sub-Committee

cc Mr J Farrell, Head of Pharmaceutical Services
1. Responsible Consultants:

Name: Professor Roy Pounder, Dr Mark Hamilton, Mr Andy Wakefield.
Department: Medicine
Name: Dr Peter Amlot
Department: Immunology

Signature denoting approval of Head of Department:

Date:.........................................

Names, qualifications and status of research workers directly involved:

Name: Dr Fiona H Gordon                Status: Clinical Research Fellow
Qualifications: MA MB BChir. MRCP(UK)

Are other Departments involved? NO

2. TITLE OF PROJECT

ANALYSIS OF IMMUNE FUNCTION IN PATIENTS WITH NEWLY DIAGNOSED INFLAMMATORY BOWEL DISEASE BY FLUORESCENCE ACTIVATED CELL SORTER ANALYSIS OF PERIPHERAL BLOOD.

3. OBJECTIVE

Hypotheses

Primary immune dysfunction is common in patients with inflammatory bowel disease and is an important aetiological factor in the disease development.

Primary immune dysfunction may predispose patients with inflammatory bowel disease to persistence of viral infections such as measles.

Detection of primary immune dysfunction in inflammatory bowel disease patients by analysis of peripheral blood lymphocytes may be masked by concomitant medication.

Aims

- To establish whether IBD patients have underlying selective immuno-deficiency by performing FACS analysis of peripheral blood lymphocytes of patients with newly diagnosed IBD, before commencing immuno-modulatory drug therapy.
- To contribute to the understanding of the immuno-pathogenesis of inflammatory bowel disease.
- To add to the understanding of the effects of immuno-modulatory drugs in vivo and to establish whether alterations in immune function noted by previous observers are truly due to IBD or are secondary to treatment.

4. DESIGN OF THE STUDY:

Peripheral blood of 40 consecutive patients who present with clinical features highly suggestive of IBD, ideally 20 Crohn’s disease (CD) patients and 20 patients with ulcerative colitis (UC), will be screened for T-lymphocyte abnormalities by FACS analysis. The proportions of a wide variety of T-lymphocyte subsets will be measured, including cells expressing activation markers, memory and naive T-lymphocytes, cytotoxic T-lymphocytes, NK cell activation markers, cells expressing VLA-4 and selectin adhesion molecules and cells expressing αβ and γδ T-cell receptor markers.

- 10ml peripheral blood will be taken at initial presentation, 1 and 4 weeks after commencing immuno-modulatory treatment and an additional blood test will be taken once in clinical remission, if this occurs after the 4 week visit.

- Clinical disease activity scores (CD; Harvey-Bradshaw, UC; Powell-Tuck) and biochemical and haematological markers of disease activity (Albumin, ESR, CRP and platelets) will be measured at each visit.

- Identical lymphocyte profiles of 20 healthy volunteer control subjects and the same haematological and biochemical markers will be measured.
Control subjects will have 20ml of peripheral blood taken for immune function, haematology and biochemical screening.

Would the samples be taken, especially for this investigation, or as part of normal patient care?

An excess of 10ml will be taken for immune function tests only

12. DISCOMFORT None

13. INSURANCE What arrangements have been made to cover the possibility of liability claims arising from this project?

Clinical Research at the Royal Free Hospital and School of Medicine.

14. COPY OF HANDOUT EXPLAINING PROJECT IN LAY TERMS TO BE GIVEN TO PATIENT OR OTHER PARTICIPANT

TITLE

Study of immune function in patients with inflammatory bowel disease

Please attach copy of handout and consent form.

(Copy of handout and consent form to be retained in patient’s hospital notes).

15. DATE OF SUBMISSION: 7th January 1998

SIGNATURE OF INVESTIGATOR: ....................................................

Please type NAME and DEPARTMENT in CAPITALS (and/or address with telephone number).

NAME: DR FIONA H GORDON

DEPARTMENT: MEDICINE (10th Floor) Ext 8057/ 3991/ 3990

16. PLEASE REPORT WHEN THE STUDY IS COMPLETED.

References


Mahida YR, et al. 5-ASA is a potent inhibitor of IL-1β production in organ culture of colonic biopsy specimens from patients with inflammatory bowel disease. Gut 1991;32:50-4.


The Royal Free Inflammatory Bowel Disease Study Group is currently investigating the role of immune dysfunction in the aetiology of inflammatory bowel disease. Preliminary work has suggested that most inflammatory bowel disease sufferers have varying abnormalities of immunity at some point during their illness, although it is unclear whether such abnormalities cause the disease itself, or whether they are secondary to long-standing inflammation.

In order to help further our understanding of inflammatory bowel disease, we would be most grateful if we could test your immune function today by taking an extra 10ml blood sample in addition to your routine tests. The test will be repeated when you are seen in 1 and 4 weeks time, in order to find out more about the effects of treatment on immune function in inflammatory bowel disease sufferers.

Please sign overleaf if you agree to take part in this study.

With many thanks,

Yours faithfully,

Dr Fiona Gordon MRCP
CLINICAL RESEARCH FELLOW

Professor Roy Pounder MD FRCP DSc
PROFESSOR OF MEDICINE

Dr Mark Hamilton MD MRCP
CONSULTANT GASTROENTEROLOGIST
19 February 1998

Dr Fiona Gordon
Department of Medicine

Dear Dr Gordon

Re: 6-98

ANALYSIS OF IMMUNE FUNCTION IN PATIENTS WITH NEWLY DIAGNOSED INFLAMMATORY BOWEL DISEASE BY FLUORESCENCE ACTIVATED CELL SORTER ANALYSIS OF PERIPHERAL BLOOD

I refer to your recent application to the Ethics Committee regarding the above project and am pleased to inform you that the project was approved at the meeting on 18th February 1998. This approval is for one year from the date of this letter. Extension of this period will be dependent on the submission of a brief synopsis of the progress of the project together with an estimation of the time required for its ultimate completion. We also require to be notified of the completion of the project and to be sent a copy of any subsequent publication.

In addition we require that:

(a) You inform the committee immediately of any information received by yourself or of any information of which you become aware which would cast doubt upon, or alter, any information contained in the original application, or any amended later application, submitted to the committee which would raise questions about the safety and/or continued contact of the research. This would include the reporting of all "adverse events" of which you become aware. These "adverse events" should also be reported to the person who provided independent review of the original application.

(b) All those involved in the study appreciate the importance of maintaining confidentiality and that they comply with the Data Protection Act 1984.

(c) All proposed amendments to the protocol, that have a bearing on the treatment or investigation of patients or volunteers, are submitted to the committee for approval.

(d) The conduct of the study complies with good clinical research practice as outlined in the ICH GCP guidelines.

(e) A copy of the patient consent form and information sheet be lodged in the clinical notes.

Yours sincerely

Dr Michael S Pegg LLM, MD, BS, BSc, FRCA
Chairman
Royal Free Hampstead NHS Trust Ethics Committee

cc Mr J Farrell, Head of Pharmaceutical Services

DOCUMENTS RECEIVED

<table>
<thead>
<tr>
<th>Document Type</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application Form</td>
<td>✓</td>
</tr>
<tr>
<td>Consent Form</td>
<td>✓</td>
</tr>
<tr>
<td>Patient Information Sheet</td>
<td>✓</td>
</tr>
<tr>
<td>GP Letter</td>
<td></td>
</tr>
<tr>
<td>Form of Indemnity</td>
<td></td>
</tr>
</tbody>
</table>
Bibliography


Antegren Publication Committee. A randomised, double-blind, placebo-controlled, pan-European study of a recombinant humanised antibody to α4 integrin (Antegren™) in moderate to severely active Crohn's disease. Gastroenterology 2001;120:G682.


Holsztynska EJ, Kung AHC, Horner HC. Pharmacokinetics of AN100226 in the

Hudson M, Chitolie A, Hutton RA, Smith MS, Pounder RE, Wakefield AJ.

Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L et al.

Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's

Ilnyckyj A, Shanahan F, Anton PA, Cheang M, Bernstein CN. Quantification of the

Ina K, Itoh J, Fukushima K, Kusugami K, Yamaguchi T, Kyokane K et al. Resistance
of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax

Irvine EJ. Usual therapy improves perianal Crohn's disease as measured by a new

orally-active inhibitor of ICAM-1 and E-selectin is efficaceous in the treatment of

Kam L, Vasiliauskas EA, Landers CJ, Targan S. Magnitude of response to Remicaide

Kane SV, Sable K, Hanauer SB. The menstrual cycle and its effect on inflammatory


Publications related to this work


Antegren Publication Committee. A randomised, double-blind, placebo-controlled, pan-European study of a recombinant humanised antibody to α4 integrin (Antegren™) in moderate to severely active Crohn's disease. *Gastroenterology* 2001;120:G682.


Practical contributions to this thesis from other workers

Clinical studies of natalizumab (Chapters 4 & 5)
I designed the protocols for these studies together with a group of investigators comprised of Professor Roy Pounder (lead investigator), Dr Steven Donoghue (Elan Pharma Ltd.), Dr Miles Allison and Dr Mark Hamilton. I was entirely responsible for conducting the trials at the Royal Free site, including obtaining local ethical approval, patient recruitment, treatment and all follow-up visits (i.e. no research nurse involved). Parallel studies of natalizumab in fifteen Crohn’s disease patients were coordinated by Dr Miles Allison and Marilyn Fouweather at the Royal Gwent Hospital, Newport. Haematological and biochemical analyses were conducted at their respective routine laboratories at the Royal Free Hospital. Natalizumab levels were performed by Athena Neurosciences Inc., South San Francisco, California, USA.

Immunological studies of natalizumab (Chapters 6 & 7) and activation antigens in IBD patients (Chapter 8)
I designed these studies together with Dr Peter Amlot, who helped with data processing and interpretation. I was entirely responsible for conducting studies of lymphocyte activation in new and chronic IBD patients, including design, ethical approval and data collection. All FACS analyses were performed by Fari Tahami and Shelly Rana in the Department of Clinical Immunology, Royal Free Hospital, London. I performed analyses of serum adhesion molecules in natalizumab-treated patients myself.
A Randomized Placebo-Controlled Trial of a Humanized Monoclonal Antibody to \( \alpha_4 \) Integrin in Active Crohn's Disease

FIONA H. GORDON, CLEMENT W. Y. LAI, MARK I. HAMILTON, MILES C. ALLISON, EMMANUEL D. SRIVASTAVA, MARILYN G. FOUWEATHER, STEPHEN DONOGHUE, CAROL GREENLEES, JAVAID SUBHANI, PETER L. AMLOT, and ROY E. POUNDER
A Randomized Placebo-Controlled Trial of a Humanized Monoclonal Antibody to α4 Integrin in Active Crohn’s Disease

FIONA H. GORDON,* CLEMENT W. Y. LAI,* MARK I. HAMILTON,* MILES C. ALLISON,* EMMANUEL D. SRIVASTAVA,* MARILYN G. FOUWEATHER,* STEPHEN DONOGHUE,* CAROL GREENLEES,* JAVAID SUBHANI,* PETER L. AMLOT, and ROY E. POUNDER*

*Centre for Gastroenterology, Department of Medicine, and iDepartment of Clinical Immunology, Royal Free and University College Medical School, London, England; *Gastroenterology Research Unit, Royal Gwent Hospital, Newport, South Wales; and §Elan Pharma Ltd., Letchworth, Hertfordshire, England

Background & Aims: α4 integrins are important mediators of leukocyte migration across vascular endothelium. This pilot placebo-controlled study aimed to assess the safety and efficacy of natalizumab, a recombinant humanized monoclonal antibody to α4 integrin, in patients with mild to moderately active Crohn’s disease.

Methods: Thirty patients with active Crohn’s disease (Crohn’s Disease Activity Index [CDAI] > 151 and < 450) received a 3-mg/kg infusion of natalizumab (n = 18) or placebo (n = 12) by double-blind randomization. The study’s primary endpoint was change in CDAI at week 2.

Results: At week 2, the CDAI decreased significantly from baseline after infusion of natalizumab (mean 45 points) but not placebo (mean 11 points). Seven (39%) natalizumab-treated patients achieved remission at week 2, compared with 1 (8%) treated with placebo. In contrast, 4 (33%) of the placebo-treated patients required rescue medication by week 2, compared with 2 (11%) natalizumab-treated patients. Significant increases in circulating B and T lymphocytes were detected only after natalizumab administration. The frequency of commonly reported adverse events did not differ significantly between groups.

Conclusions: A single 3-mg/kg natalizumab infusion was well tolerated by Crohn’s disease patients, although the dose used may have been suboptimal. Elevated circulating lymphocyte levels after natalizumab suggest interrupted lymphocyte trafficking. Natalizumab therapy in active Crohn’s disease merits further investigation.

Integrins are heterodimeric glycoproteins that are widely expressed on leukocytes and are thought to be important mediators of leukocyte adhesion to vascular endothelium. The α4 integrin is expressed at a moderate or high level on almost all lymphocytes and to a lesser extent on monocytes and eosinophils. α4 integrins usually exist in combination with either a β1 or β7 subunit and interact predominantly with the endothelial ligands vascular cellular adhesion molecule 1 (VCAM-1) and mucosal addressin cellular adhesion molecule (MAdCAM-1), respectively.

Human and animal studies have suggested that the interaction between α4β7 and MAdCAM-1 is particularly important in mediating leukocyte homing to gut mucosa. Furthermore, studies of patients with inflammatory bowel disease (IBD) show that endothelial cells extracted from inflamed intestinal mucosa demonstrate increased α4-dependent adhesiveness to leukocytes in vitro.

The hypothesis that α4 integrin blockade could be useful therapy for IBD stems mainly from animal studies. In particular, monoclonal antibody blockade of α4 integrins, either alone or in combination with β7, has been shown to produce resolution of the spontaneous colitis of captive cotton-topped tamarins. Clinical trials of such antibodies in Crohn’s disease have not been performed to date, although results of a pilot study of a humanized antibody to α4β7 integrin are promising in patients with ulcerative colitis.

Natalizumab (Antegren; Elan Pharma Ltd., Letchworth, England) is a recombinant humanized antibody that has been derived from a murine monoclonal antibody (AN100226m) raised against human α4 integrin. AN100226m was humanized by complementarity-determining region grafting of the hypervariable region of the gene encoding AN100226m onto a human immunoglobulin (Ig) G4 framework. The resultant antibody contains approximately 5% mouse-derived protein only. In vitro, natalizumab has been shown to block the ad-
hension of human Jurkat α4β1-expressing cells to high-density purified recombinant VCAM-1 and of α4β7-expressing RPMI-8866 cells to recombinant MAdCAM-1 (Yednock TA, personal communication, May 1999). It also produces clearance of leukocytes in the central nervous system of guinea pigs with experimental allergic encephalomyelitis, a disease thought to be mediated by the interaction of leukocyte α4β1 integrin and VCAM-1. A multicenter study of natalizumab in patients with active multiple sclerosis and a small phase I study in 26 healthy male volunteers have shown that a single 3-mg/kg intravenous dose is safe and well tolerated.12,13

The aim of this study was to assess the efficacy and safety of natalizumab in patients with mild to moderately active Crohn's disease in a randomized, double-blind, placebo-controlled trial.

**Patients and Methods**

**Patients**

Thirty-five patients with mild to moderately active Crohn's disease, defined by a Crohn's Disease Activity Index (CDAI) score ≥151 and ≤450,14 were assessed for trial eligibility at an outpatient visit at least 1 week before the planned treatment date. Patients included in the trial were at least 18 years old and had Crohn's disease confirmed by 2 or more of the following diagnostic criteria at least 3 months before study entry: history, radiologic or endoscopic intestinal appearance, histology, presence of Crohn's-related fistula(e), and abscess formation.15 Female participants were required to have a negative pregnancy test result at study entry and to use effective contraception throughout the study follow-up period. Patients receiving mesalamine-derived drugs or azathioprine/6-mercaptopurine were eligible if that treatment had not been altered within 2 weeks of study entry. Likewise, patients receiving oral corticosteroids (≥40 mg prednisolone or ≥9 mg budesonide daily) were included if the dose had not been within 2 weeks of study entry. Patients kept a daily symptom diary card for at least 7 consecutive days before each outpatient visit which, together with examination and laboratory findings, was used to calculate their CDAI scores. Remission was defined as a CDAI score ≤155, using a paired t-test with 5% 2-sided significance level. The number of natalizumab-treated patients was increased to 20 to allow for early withdrawals and provide sufficient preliminary safety data. It was our intention that patients should be assigned treatment with natalizumab or placebo in a 2-to-1 randomization, i.e., 20 natalizumab to 10 placebo. However, a randomization error at one center (Royal Free Hospital) resulted in eventual active treatment and placebo groups of 18 and 12 patients, respectively (Figure 1). Individual randomization concealment codes were held by the trial's sponsor and each hospital's pharmacy for emergency use, and none were opened during the study. Investigators and patients remained blinded to the randomization codes until data analysis was complete.

Patients received a single 3-mg/kg intravenous infusion of natalizumab (Antegren) or placebo over 30–75 minutes. Natalizumab (5 mg/mL) was formulated in a solution of 50 mmol/L histidine buffer and 0.02% polysorbate 80 adjusted to pH 6 with hydrochloric acid and was diluted to 100 mL in 0.9% saline for administration. Placebo consisted of 10 mmol/L phosphate-buffered 0.02% polysorbate 80 similarly diluted to 100 mL in 0.9% saline. Patients were kept under direct observation for a minimum of 6 hours from the start of the infusion.

Patients were reviewed 1, 2, 4, 8, and 12 weeks after treatment. Patients kept a daily symptom diary card for at least 7 consecutive days before each outpatient visit which, together with examination and hematologic findings, was used to calculate their CDAI scores.
score of <150. Rescue medication was defined as the initiation or increased daily dose of any of the following drugs: corticosteroids (intravenous or oral), mesalamine, or azathioprine/6-mercaptopurine. Quality of life was assessed by the IBD questionnaire (IBDQ) completed before and 4 weeks after treatment. Venous blood was taken at each visit for analysis of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), full blood count, serum biochemical screening (including pregnancy tests in women), and serum natalizumab and antinatalizumab antibody concentrations. The proportion of peripheral blood T cells (TCRαβ+) and B cells (CD19+) were also measured at all follow-up visits using fluorescence-activated cell-sorter (FACS) analysis (Becton Dickinson, Oxford, England) as described previously.

Statistical Analysis

The change in CDAI at 2 weeks postinfusion in the intention-to-treat population was used as the study endpoint and was analyzed using a 2-tailed paired t test (P < 0.05). Secondary endpoints included the percentage of patients in remission (CDAI < 150), and the percentage who required rescue medication at 2 weeks postinfusion. To try to account for improvements in CDAI score because of rescue medication, the last CDAI value recorded before administration of rescue medication was carried forward for statistical analyses at subsequent time points (i.e., last observation carried forward, or LOCF data). LOCF data were also used for patients who were withdrawn from the study.

Paired and unpaired t tests were used for within-group and between-groups analyses of parametric data, respectively. Wilcoxon signed rank and Mann–Whitney tests were used for equivalent analyses of nonparametric data. The χ² test was used to test for differences in remission rates between groups.

### Results

#### Demography

The demographic characteristics of the 30 patients who received natalizumab or placebo are shown in Table 1. Five patients were screened but not included in the study because of blood test abnormalities or personal choice. The groups were well matched for age, sex, height, ethnicity, disease duration, and IBDQ score, although mean pretreatment weight was greater in the natalizumab group (P = 0.02). The mean pretreatment CDAI of the natalizumab group (258 points) was less than that of placebo (273 points), but the difference was not significant (P = 0.95). One natalizumab group patient had a pretreatment CDAI of 122 points, accounted for by a combination of an unexpected increase in hematocrit and calculation error in one subsection of the CDAI. This was not recognized until after treatment, and the patient’s data were therefore included in all analyses. At study entry, the mean prednisolone dose (12 mg in natalizumab group; 15 mg in placebo group) and the proportions of patients receiving mesalamine or azathioprine/6-mercaptopurine did not differ significantly between groups. The flow of patients through the trial is shown in Figure 1.

#### Clinical Response to Treatment

At 2 weeks, natalizumab-treated patients had a statistically significant mean reduction in CDAI of 45 points (P = 0.02), i.e., from 258 (range, 122–436) to 213 (range, 46–413) points, although this is not significantly different from the mean 11-point change that occurred in placebo-treated patients (P = 0.2; Figure 2). At 4 weeks, the reduction in CDAI in natalizumab-treated patients remained significant (P = 0.01), but by this time many patients had begun rescue therapy (9 [50%] of 18 in the natalizumab group, 8 [67%] of 12 in the placebo group), hence LOCF data were used for these patients. In placebo-treated patients, the change in mean CDAI from baseline values at weeks 2 and 4 was not statistically significant. By 2 weeks, 39% (n = 7) of the 18 patients who received natalizumab had experienced remission, compared with 8% (n = 1) of the placebo group (P = 0.1; Figure 3; observed differences between groups not statistically significant). Remission was sustained to at least week 12 in 2 of the natalizumab-treated patients; the rest required rescue therapy a median of 22 weeks.

---

**Table 1.** Demographic Characteristics of Patients at Study Entry

<table>
<thead>
<tr>
<th>Characteristic, mean (SD)</th>
<th>Placebo (n = 12)</th>
<th>Natalizumab (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease duration (yr)</td>
<td>8.4 (6.0)</td>
<td>8.5 (9.6)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>34.4 (8.8)</td>
<td>36.0 (13.2)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>5/7</td>
<td>7/11</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.5 (8.6)</td>
<td>66.4 (10.6)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>161.5 (7.1)</td>
<td>165.5 (8.7)</td>
</tr>
<tr>
<td>Ethnicity (white/Asian)</td>
<td>11/1</td>
<td>17/1</td>
</tr>
<tr>
<td>Disease site, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal or ileocecal alone</td>
<td>5 (42)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>Colonic alone</td>
<td>3 (25)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Ileal and colonic</td>
<td>4 (33)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Perianal</td>
<td>4 (33)</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Activity, mean (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDAI</td>
<td>273 (191–420)</td>
<td>258 (122–436)</td>
</tr>
<tr>
<td>IBD quality-of-life score</td>
<td>118 (78–144)</td>
<td>121 (74–167)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>35 (42)</td>
<td>14 (12)</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>42 (4)</td>
<td>41 (3)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>27.5 (12.9)</td>
<td>26.4 (13.4)</td>
</tr>
<tr>
<td>Medication at week 0, n (%)</td>
<td>1 (8)</td>
<td>3 (17)</td>
</tr>
<tr>
<td>None</td>
<td>1 (8)</td>
<td>3 (17)</td>
</tr>
<tr>
<td>Prednisolone/budesonide</td>
<td>9 (75)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>2 (17)</td>
<td>6 (33)</td>
</tr>
<tr>
<td>Mesalamine</td>
<td>9 (75)</td>
<td>13 (72)</td>
</tr>
<tr>
<td>Mesalamine alone</td>
<td>2 (17)</td>
<td>3 (17)</td>
</tr>
</tbody>
</table>
days after infusion (range, 17–89 days). Overall, by 2 weeks, rescue treatment had been begun in 33% (n = 4) of placebo-treated patients, compared with 11% (n = 2) of those who received natalizumab (Figure 3). Finally, 50% (n = 9) of natalizumab-treated patients and 59% (n = 7) of placebo-treated patients continued to have active Crohn’s disease at 2 weeks but had not begun rescue therapy.

Quality of Life

A significant improvement in mean IBDQ score occurred from 121 points at baseline to 140 points at 4 weeks in patients who received natalizumab (P = 0.004). No significant improvement in IBDQ score was observed in placebo-treated patients.

Inflammatory Markers

Patients who received natalizumab experienced a significant reduction in CRP at 2 and 4 weeks compared with baseline levels (P = 0.02 at both time points), and a significant reduction in ESR at 4 weeks only (P = 0.04). These changes did not differ significantly from those in the placebo group. In placebo-treated patients, however, the changes from baseline values in CRP and ESR at 2 and 4 weeks were not significant. Platelet counts did not change significantly in either treatment group between baseline and 4 weeks. Finally, there were no significant differences between groups in any of these inflammatory parameters at any point in the study.

Pharmacokinetics and Antibody Formation

The mean plasma half-life of natalizumab was 4.8 days (Figure 4). The mean maximum serum concentration (Cmax) achieved at 1 hour postinfusion was 52.8 μg/mL, and most patients had detectable serum levels of natalizumab at 4 weeks, with a mean serum concentration of 0.99 μg/mL. Two patients (11%) developed transient low-titer non–anti-idiotypic antibodies to natalizumab, detectable only at a single visit during the 12-week trial follow-up period. One of these patients experienced symptomatic remission from week 1 to at least week 12, developing detectable antinatalizumab antibodies at week 8. Antibodies to natalizumab were detected at week 4 in the other patient, who showed no clinical response to natalizumab.

Tolerability and Adverse Events

The infusion was generally very well tolerated. The most common adverse events, reported in at least 20% of patients during the 12-week follow-up period, were headache (50% of each treatment group), Crohn’s disease (natalizumab, 39%; placebo, 42%), and abdominal pain (natalizumab, 22%; placebo, 17%). The frequency of these events did not differ significantly

Figure 2. Mean CDAI by patient group (LOCF data), weeks 0–4. Bars indicate SD, and numbers above and below indicate number of patients in each group who had begun receiving rescue therapy.

Figure 3. Clinical status of patients 2 weeks after a single infusion of 3 mg/kg natalizumab or placebo at 2 weeks postinfusion. Observed differences between groups are not significant.

Figure 4. Mean serum natalizumab concentrations of 18 patients after a single 3-mg/kg infusion. Bars indicate SD.
between groups. However, 6 natalizumab-treated patients required admission because of problems related to Crohn's disease, namely worsening of symptoms in 5 patients and chronic anemia requiring blood transfusion in 1 patient. Two of the patients admitted for symptomatic relapse had initially achieved remission at 2 weeks postinfusion, 1 had experienced a CDAI reduction of 70 points at 1 week, and the other 2 had not responded to natalizumab. Overall, the median time from natalizumab infusion to admission was 48 days and ranged from 16 to 66 days. Two natalizumab-treated patients (included in the admission group above) and 1 placebo-treated patient required surgical resection of Crohn's disease at 66, 69, and 70 days, respectively. Two of these patients (1 natalizumab, 1 placebo) were withdrawn from the study, and another placebo-treated patient was withdrawn after failing to attend follow-up visits beyond 4 weeks postinfusion. All 3 withdrawals occurred at least 50 days after the study's start; therefore, these patients' data were included in the primary endpoint analyses at 2 weeks.

**Immunologic Parameters**

A significant increase in mean circulating lymphocyte counts occurred 1, 2, and 4 weeks postinfusion in natalizumab-treated patients only (Figure 5; $P < 0.01$ for each time point compared with baseline). FACS analysis showed that this increase included both B-cell (CD19+) and T-cell (TCR$\alpha\beta$+) subsets.

**Discussion**

This study shows that treatment with a single 3-mg/kg infusion of natalizumab (Antegren) is well tolerated by patients with active Crohn's disease. Remission occurred 2 weeks postinfusion in a greater proportion of natalizumab-treated patients than of those who received placebo, but the difference between groups was not significant. Natalizumab-treated patients achieved significant reductions in CDAI 2 and 4 weeks after treatment compared with baseline, although results of between-groups comparisons with placebo-treated patients were not statistically significant. However, CDAI reductions in natalizumab-treated patients were accompanied by improvements in CRP, ESR, and IBDQ quality-of-life scores, suggesting a positive efficacy trend.

The effects of natalizumab at a dose of 3 mg/kg appear to be relatively short-lived in patients with active Crohn’s disease. This is suggested by the finding that 5 of the 7 patients who initially achieved remission at 2 weeks had subsequent relapses and required rescue therapy at a median of 22 days postinfusion. This finding may be related to the finding that the half-life of natalizumab is 4.8 days in IBD patients, shorter than the 8.7 days observed in healthy volunteers. Additionally, in vitro leukocyte-saturation studies now suggest that a minimum serum concentration of approximately 3 $\mu$g/mL of natalizumab is required to produce appropriate saturation of at least 80% of membrane-bound $\alpha_4$ integrins (Elan Pharma Ltd, unpublished data, June 1999). The mean serum concentrations of natalizumab at 2 and 4 weeks postinfusion were 4.91 and 0.99 $\mu$g/mL, respectively, suggesting perhaps that blockade of $\alpha_4$ integrins was suboptimal after a single 3-mg/kg dose and that larger and/or more frequent doses might result in improved efficacy.

It is unclear why the half-life of natalizumab was shorter in Crohn’s disease patients than in healthy volunteers. Patients in our study may have had a higher proportion of circulating $\alpha_4^+$ cells than healthy volunteers, although previous studies of peripheral blood $\alpha_4^+$ T cells suggest that this is unlikely. The serum half-life of a recombinant humanized anti-TNF-\(\alpha\) antibody (CDP571) was also found to be shorter in Crohn’s patients than that predicted by a phase I study. This may be related to the higher tissue and/or circulating TNF-\(\alpha\) concentrations found in IBD patients than in unaffected individuals.

All serious adverse events recorded during the study were caused by worsening of underlying Crohn’s disease as a consequence of lack of sustained efficacy and were therefore considered unrelated to natalizumab itself. The low-titer antibodies formed by 2 patients
The effects on lymphocytes are consistent with effects on circulating lymphocytes and inflammatory mediator ablation, which produced only short-lived reduction in symptoms and remission of patients with multiple sclerosis developed transient low-titer antibodies after receiving 2 infusions of 3 mg/kg natalizumab, 1 month apart, in a recent multicenter study. An increase in both B and T cells accounts for the transient but significant lymphocytosis detected in natalizumab-treated patients alone. This effect appears to be consistent after infusion of monoclonal antibodies to certain adhesion molecules in humans and in animal models. For example, lymphocytosis was seen after infusion of monoclonal antibodies either to α4 alone or to α4β7 integrin in the cotton-topped tamarin. Furthermore, a clinical trial of monoclonal antibody to ICAM-1 also produced transient elevation of lymphocyte counts in patients with rheumatoid arthritis. A possible explanation for this finding in our study is that natalizumab selectively inhibits α4 integrin-mediated lymphocyte migration through vascular endothelium. Thus, α4+ lymphocytes are forced to remain in the intravascular compartment, and lymphocyte trafficking is interrupted. Although inflammation in IBD is thought to be mediated predominantly by T cells, the transient increase in B cells is likely to be related to the finding that α4 integrins are highly expressed on these cells.

In conclusion, treatment of mild to moderately active Crohn's disease with blockade of α4 integrin by natalizumab (Antegren) did not show significant efficacy compared with placebo in this pilot study. However, the single dose of 3 mg/kg was probably suboptimal because it produced only short-lived reduction in symptoms and effects on circulating lymphocytes and inflammatory mediators. The effects on lymphocytes are consistent with those in similar animal studies and support the hypothesis that lymphocyte trafficking to the human gut is mediated by α4 integrins. Further work is needed to establish the optimal dose level and dosing regimen required to assess the potential therapeutic benefit of natalizumab in the treatment of patients with active Crohn's disease.

References


Received October 20, 2000. Accepted April 5, 2001.

Address requests for reprints to: Fiona H. Gordon, M.A., M.B.B.Chir., M.R.C.P., Centre for Gastroenterology (10th Floor), Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, England. e-mail: fionagordon@ownhomeline.freeserve.co.uk; fax: (44) 20-7431-5261.

Supported by Elan Pharmaceuticals Inc., South San Francisco, California.

The authors thank Dr. Bruce MacFarlane and Dr. Alistair McNair for kindly referring their patients and Tanya Palmer, Judy Sercombe, Shelley Rana, and Farl Tahami for technical assistance.