The effect of interleukin-10 and of interleukin-12 on the anti-double-stranded DNA antibody production by the blood lymphocytes of patients with systemic lupus erythematosus

by

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Abstract

The effect of interleukin-10 and of interleukin-12 on the anti-double stranded DNA antibody production by the blood lymphocytes of patients with systemic lupus erythematosus.

Systemic lupus erythematosus (SLE) is a chronic autoimmune rheumatic disease, characterised by B-cell hyperactivity and the presence of various autoantibodies. Anti-double stranded DNA antibodies (anti-dsDNA antibodies) are thought to be important in SLE pathogenesis as their serum titre often correlates with disease activity and they have been shown to induce lupus-like organ damage in many experimental models.

If anti-dsDNA antibodies in patients with SLE were pathogenic, then the immune signals responsible for their expansion could be responsible for inducing disease flare. The cytokines interleukin-10 (IL-10) and interleukin-12 (IL-12) regulate antibody production in other systems and the levels of both of these cytokines are related to disease activity in SLE. Serum levels of IL-10 correlate positively with disease activity and the in vitro production of IL-12 correlates negatively with disease activity, moreover this deficit in IL-12 production is due to IL-10 overproduction. This observation raises the possibility that the imbalance between these two cytokines might be involved in anti-dsDNA antibody production.

Cultures of blood mononuclear cells from SLE patients with different disease activities were treated with IL-10 or IL-12 and the resulting supernatants tested for their antibody production by ELISA; both cross-sectional and longitudinal studies were carried out. The results showed that the effect of IL-10 on antibody production
varied with disease activity. Further experiments using depleted cell populations revealed that T-cells, but not monocytes, played a role in determining the effects of IL-10. Parallel experiments utilising flow cytometry revealed that IL-10 may exert some of its T-cell dependent effects on antibody production through the induction of apoptosis. IL-12 both increased and decreased anti-dsDNA antibody production and a number of different factors, including disease activity and inter-patient variation might be responsible for this.

These findings are consistent with the role of IL-10 in the regulatory suppression of immune responses. The effect of IL-12 requires further examination.
Dedication

To Pauline, Rodney and Hema
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor David Isenberg and Professor Peter Lydyard, for the regular meetings to guide my progress and for their patience through the various stages of writing-up. I was extremely fortunate to work for two such approachable and able supervisors.

I would like to thank my colleagues in the lab who contributed to the work in this thesis. Lesley Mason for her help in tissue culture, Deborah McNab for the ELISA she did and Dr. CT Ravirajan for the use of his anti-IL-10 monoclonal antibody.

I would also like to thank all the members of the lab for their support and advice; it was a pleasure working with you. Thanks to colleagues Ajay, Arthi, Barry, Emma, Gilly, Jose, Lesley, Liz, Matt, Rizgar, Sam, Selina, Tindie, Tracey, Vikki, Dr. Jo Cambridge and Dr. CT Ravirajan.

I would particularly like to thank Arthi Anand for all her help and support.

Thanks to the secretaries Kate and Pam, for always knowing where my supervisors were and always smiling when I arrived with the latest bundle of paper.
Thanks to UCL for granting me the Emily de Rossignol fund to complete the work in this thesis and for accepting me onto the MB Ph.D. programme; thank you Professor AW Segal, Professor N Woolf, Dr G Stewart and Mr. T Wale.

I would also like to thank all the patients who kindly donated their blood to this project.

DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.
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Abbreviations

Ab – Antibody
ANA – Anti-nuclear antibody
APC – Antigen presenting cell
BIC – Bicarbonate buffer
C – Complement component
CDR – Complementarity determining region
DNA – Deoxyribonucleic acid
Ds – Double stranded
EDTA – Ethylenediaminetetra – acetic acid
ELISA – Enzyme linked immunosorbant assay
Fab – Antigen binding fragment of antibody
Fc – Crystallisable fragment of antibody
FCS – Fetal calf serum
GBM – Glomerular basement membrane
HC – Healthy control
HS – Heparin sulphate
Ig – Immunoglobulin
IgG – Immunoglobulin class G
IgM – Immunoglobulin class M
IL – Interleukin
l - litres
mg - Milligrammes
ml – millilitres
mMol - millimoles
ng - nanogrammes
OD – optical density
PBS – Phosphate buffered saline
PBMC - Peripheral blood mononuclear cells
MHC – Major histocompatibility complex
SCID – Severe combined immunodeficiency
sIg – surface immunoglobulin
ss – single stranded
TGF – Transforming growth factor
TNF – Tumour necrosis factor
Chapter 1

Introduction

1.1.1 Definition of systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune rheumatic disease with no known cure (Oxford Textbook of Rheumatology, second edition). The American College of Rheumatology (ACR) classification criteria were originally formulated to classify the heterogeneous population of SLE patients; they are widely used as a diagnostic criteria. A patient can be diagnosed with SLE if they serially or simultaneously exhibit four or more of these criteria. This gives a correct definition of SLE with 95% sensitivity and 85% specificity (Hahn 1998). The latest criteria are shown in table 1.1 (next page).

1.1.2 Prevalence of SLE

The peak age of onset of SLE is between the ages of 20 and 40; it affects women more than men (the relative ratio being approximately 9:1) and some racial groups more than others. In the Birmingham, UK area (circa 1995), the prevalence of SLE in females was 20.6:10,000 in Afro-Caribbeans, 9.1:10,000 in Asians and 3.6:10,000 in Caucasians (Johnson et al. 1995). The equivalent ratios for males were 0.93:10,000,
2.6:10,000 and 3.4:10,000 respectively. Another group who sampled in Europe (Fessel et al. 1974) has described similar ratios.

Table 1.1 The ACR criteria

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Malar rash</td>
<td>Fixed malar erythema</td>
</tr>
<tr>
<td>2) Discoid rash</td>
<td>Erythematous raised patches</td>
</tr>
<tr>
<td>3) Photosensitivity</td>
<td>Skin rash as an abnormal reaction to sunlight</td>
</tr>
<tr>
<td>4) Oral ulcers</td>
<td>Ulcers are usually painless and observed by the physician</td>
</tr>
<tr>
<td>5) Arthritis</td>
<td>Non-erosive arthritis affecting two or more peripheral joints</td>
</tr>
<tr>
<td>6) Serositis</td>
<td>Either pleuritis or pericarditis for which convincing clinical evidence exists</td>
</tr>
<tr>
<td>7) Renal disorder</td>
<td>Persistent proteinuria &gt; 0.5g/day or cellular casts on urine microscopy</td>
</tr>
<tr>
<td>8) Neurological disorder</td>
<td>Seizures or psychosis in the absence of other causes.</td>
</tr>
<tr>
<td>9) Blood disorder</td>
<td>Haemolytic anaemia or leukopenia &lt; 4000/mm$^3$ or lymphopenia &lt; 1500/mm$^3$ or thrombocytopenia &lt; 100000/mm$^3$ (non-drug induced)</td>
</tr>
<tr>
<td>10) Immunological disorder</td>
<td>Positive anti-double stranded DNA or anti-Sm or positive finding of anti-phospholipid antibodies</td>
</tr>
<tr>
<td>11) Anti-nuclear antibody</td>
<td>Abnormal titre of ANA in the absence of drugs known to be associated with “drug induced lupus”</td>
</tr>
</tbody>
</table>

Tan et al. 1982 and Hochberg et al. 1997
1.1.3 Clinical assessment of SLE

SLE is a chronic disease, the activity of which varies over time such that patients can sometimes be in full remission. Various scales have been devised to monitor these changes in disease activity. The British Isles lupus assessment group (BILAG) score was used to assess each patient included in this thesis (Hay *et al.* 1993). This index is based on the physician’s intention to treat principle and divides lupus activity into eight organs or systems (general, skin, central nervous system, musculoskeletal, cardiovascular/respiratory, vascular, renal and haematological) and grades each one. The most active state, implying the need for major immunosuppressive therapy, is given an A grade, while the absence of activity in that system receives an E. To convert these individual organ grades into a global score, the following system was used: - A=9, B=3, C=1 and D/E=0. A patient with a global score of more than or equal to 6 is regarded as having active disease and 0-5 relatively inactive disease. The BILAG questionnaire is included in the appendix.

Many other disease activity scores exist: the European consensus lupus activity measure (ECLAM), systemic lupus erythematosus disease activity index (SLEDAI) systemic lupus activation measure (SLAM) and Lupus activity Index (LAI). These, however, are global scores which necessarily provide a relatively crude assessment of disease activity. Ward *et al.* (2000), used the above scores to assess a cohort of 19 patients (who were reviewed fortnightly for 40 weeks) showing that there was a statistically significant correlation between the results obtained from each score. During that study, 16 of the patients experienced a 96% or greater change in their disease activity as measured by the BILAG score.
1.1.4 Clinical features of SLE

As can be seen from table 1.1 it is possible for two patients, both diagnosed with SLE, not to share a single symptom. It is therefore important to state the prevalence of each clinical feature when defining SLE; this is done in table 1.2, which presents approximate cumulative data from 300 patients with SLE followed up in the UCH/Middlesex series between 1978 and 2000. SLE not only causes morbidity, but is also the cause of mortality. Forty-one of these 300 patients are known to have died. The causes of death are divided into four approximately equal groups: vascular, infection, malignancy and miscellaneous (ranging from “old-age” to renal). The age at death ranged from 16-87 years and the average age of death of this subset was 51 years (Isenberg D, personal communication).

1.2 Aetiopathogenesis of SLE

It is thought that a number of hormonal, genetic and environmental factors combine to predispose certain individuals to SLE and precipitate the occurrence of disease (Kotzin 1996). These factors are discussed in detail elsewhere and are not described here.

1.3 Autoantibodies in SLE

A likely cause of the clinical manifestations seen in SLE are autoantibodies (Kotzin 1996), evidence supporting this will be presented later. Most of these autoantibodies are against nuclear extracts (anti-nuclear antibodies [ANA]). Cervera et al (1993) studied 1000 patients with SLE, 96% had anti-nuclear autoantibodies and 78% had
Table 1.2 Cumulative prevalence of clinical features of patients with SLE.

<table>
<thead>
<tr>
<th></th>
<th>Approximate cumulative prevalence (%)</th>
<th>Approximate cumulative prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Musculoskeletal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthralgia/Arthritis</td>
<td>90</td>
<td>Depression</td>
</tr>
<tr>
<td>Tenosynovitis</td>
<td>20</td>
<td>Psychosis</td>
</tr>
<tr>
<td>Myalgia</td>
<td>50</td>
<td>Seizures</td>
</tr>
<tr>
<td>Myositis</td>
<td>5</td>
<td>Hemiplegia</td>
</tr>
<tr>
<td><strong>Cardiopulmonary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>40</td>
<td>Cerebral</td>
</tr>
<tr>
<td>Pleurisy</td>
<td>35</td>
<td>Depression</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>25</td>
<td>Psychosis</td>
</tr>
<tr>
<td>Lupus pneumonitis</td>
<td>5</td>
<td>Migraine</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>5</td>
<td>Anaemia (Iron deficiency)</td>
</tr>
<tr>
<td>Pulmonary function</td>
<td>85</td>
<td>Anaemia (Chronic disease)</td>
</tr>
<tr>
<td>abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiomegaly</td>
<td>20</td>
<td>Autoimmune haemolytic anaemia</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>15</td>
<td>Leucopenia</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>10</td>
<td>Lymphopenia</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>5</td>
<td>Anaemia (Chronic disease)</td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td>40</td>
<td>Circulating anti-coagulants</td>
</tr>
<tr>
<td>Nausea</td>
<td>15</td>
<td>Dermatological</td>
</tr>
<tr>
<td>Vomiting</td>
<td>&lt; 10</td>
<td>Butterfly rash</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>&lt; 10</td>
<td>Erythematous maculopapular eruption</td>
</tr>
<tr>
<td>Asciites</td>
<td>&lt; 10</td>
<td>Discoid lupus</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>30</td>
<td>Relapsing nodular non-suppurative panniculitis</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>25</td>
<td>Vasculitic skin lesions</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>10</td>
<td>Livedo reticularis</td>
</tr>
<tr>
<td><strong>Renal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haematuria</td>
<td>10</td>
<td>Purpuric lesions</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>60</td>
<td>Alopecia</td>
</tr>
<tr>
<td>Casts</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Serum albumin &lt; 35 g/l</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine &gt; 125 mmol/l</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Reduced 24-hour creatinine clearance</td>
<td>35</td>
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</tbody>
</table>

raised titres of anti-double stranded DNA antibodies (anti-dsDNA antibodies),
making anti-dsDNA the most common type of ANA (see table 1.3). In contrast to
anti-single stranded DNA (anti-ssDNA antibodies), anti-dsDNA antibodies are very
rarely found in any disease other than SLE (Koffler et al 1971, Isenberg et al. 1985).
Together these observations provide two reasons that make anti-dsDNA antibodies
attractive antibodies for research: first their high prevalence and specificity to SLE
suggest that the may be important in SLE pathogenesis and second, their high
prevalence make them relatively easy to study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>% prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-nuclear antibody</td>
<td>96</td>
</tr>
<tr>
<td>Anti-Ro</td>
<td>25</td>
</tr>
<tr>
<td>Anti-La</td>
<td>19</td>
</tr>
<tr>
<td>Anti-Sm</td>
<td>10</td>
</tr>
<tr>
<td>Anti-U1RNP</td>
<td>13</td>
</tr>
<tr>
<td>IgG Anti-cardiolipin</td>
<td>24</td>
</tr>
<tr>
<td>IgM Anti-cardiolipin</td>
<td>13</td>
</tr>
<tr>
<td>Anti-C1q *</td>
<td>33*</td>
</tr>
<tr>
<td>IgG Anti-dsDNA</td>
<td>78</td>
</tr>
</tbody>
</table>

Adapted from Cervera et al. (1993) and Walport * et al (1998)

1.3.2 Characterisation of the class of anti-DNA antibodies in patients with SLE

Antibodies vary by class and binding profile. Zouali et al. (1984) tested the serum of
44 patients and 50 healthy controls (HC) and found no differences in the proportions
of the four IgG subclasses, the absolute level of IgG was not presented; though it
would be expected to be higher than in HC. There were IgM and IgG anti-dsDNA antibodies of all the IgG subclasses, however the vast majority of anti-dsDNA antibodies were IgG1 or IgG3 (the complement binding IgG subclasses). IgG anti-ssDNA antibodies were equally prevalent in each subclass.

Deng et al. (1990) tested the serum of 27 lupus patients with subacute cutaneous lupus erythematosus and 29 patients with SLE. It was found that ANA binding was confined to the IgG1 subclass in subacute lupus while all four subclasses had ANA binding activity in the systemic form of the disease.

### 1.3.3 Binding characteristics of anti-dsDNA antibodies from patients with SLE

Anti-dsDNA antibodies may also vary by specificity, in that they may bind dsDNA only or a combination of dsDNA and other molecules. Of the monoclonal anti-dsDNA antibodies that have been studied, very few bind only double-stranded DNA, most bind ssDNA and other molecules such as heparin sulphate as well (Hahn 1998).

### 1.3.4 Correlation between disease activity and anti-dsDNA antibody titre

Clearly the existence of a chronological association between disease activity and anti-dsDNA antibody titre would add weight to the argument that these antibodies are one of the pathogenic factors in SLE. The existence of such an association is still somewhat controversial with some studies finding a strong correlation, particularly
with vasculitis and glomerulonephritis (Ravirajan et al. 2001), while others find only a weak association (Esdaille et al. 1996; Petri et al. 1991). This disparity in results may be due to the detection method used (see section 1.3.4a) or missing the expansion in antibody levels as they are sequestered to the target organ, e.g. kidney (see section 1.3.4b). Alternatively in those patients tested, there may simply be no association (see section 1.3.4c). These possibilities will now be discussed.

1.3.4a Detection method

There are a number of different methods that can be used to detect anti-dsDNA antibodies; these have been reviewed in detail recently (Isenberg and Smeenk 2002). However, a detailed explanation of each method is beyond the scope of this introduction. Therefore, to illustrate the point that the assay system used can affect the results gained; the data obtained from just two different methods (the Farr assay and the anti-dsDNA ELISA) will be described. The Farr assay will only detect antibodies that bind dsDNA with high affinity; this detection method produces results that correlate best with disease activity and the presence of glomerulonephritis (Smeenk et al 1991; ter-Borg et al. 1990). However, the Farr assay is more technically challenging than the ELISA (the Farr assay requiring radioactivity) therefore the ELISA has come to dominate the literature. The anti-dsDNA antibody ELISA will detect antibodies of high and low affinity. These observations imply firstly, that the lower affinity antibodies are not pathogenic. Secondly, that patients may have consistently elevated levels of anti-dsDNA antibodies as measured by ELISA, but
low disease activity because the immune system has not yet made a high affinity, pathogenic, anti-dsDNA antibody. (This possibility will be further discussed in 1.3.4b)

1.3.4b Antibody sequestration

To obtain accurate data regarding the loss of serum antibody into the kidneys (or other organs), longitudinal studies need to be undertaken. Williams et al. (1999) followed 48 patients, of which 27 had renal involvement for between two and eight years. An ELISA was used to measure the serum anti-dsDNA antibody titre of these patients at each visit. During bouts of renal disease some loss of serum anti-dsDNA antibody titre was demonstrated, while those patients without renal disease showed no such variation. A better predictor of renal flare appeared to be antibody affinity, with the loss of high affinity anti-dsDNA and sometimes anti-nucleosome antibodies correlating with renal involvement. In 10 patients with lupus nephritis the renal eluates were also measured for anti-DNA binding affinity and it was discovered that after a sudden exacerbation in lupus nephritis there was a significantly higher affinity of DNA binding in the eluate than in the serum. Koffler et al. (1967) took cadaveric renal samples from 10 patients who had lupus nephritis and one from a patient who had a histologically similar nephritis that was unrelated to SLE. Those patients who died of lupus nephritis had plentiful glomerular deposits of IgG and complement but less IgM. Elution of this glomerular antibody showed it had a higher content of ANA binding than the serum and that the ANA binding could be inhibited by dsDNA. The
patient with nephritis not related to SLE did not show ANA activity in the glomerular eluate. Together these data show that anti-dsDNA antibodies are not likely to be the consequence of nephritis; though only one patient with nephritis of another cause was tested. The enrichment of anti-dsDNA antibodies in the eluate as compared to the serum argues against their deposition being a non-specific event.

1.3.4c No association

It has been shown that some patients maintain high titres of IgG anti-dsDNA antibodies without ever suffering lupus nephritis (Gladman et al. 1979). This may be due to a lack of a high affinity, pathogenic antibody or the absence of some hitherto undefined “second hit”. At the other end of the spectrum, a very small number of patients have lupus nephritis without detectable anti-dsDNA antibodies (Maddison and Reichlin 1979; Maddison et al. 1981). There is a subset of patients with renal disease who have been shown to have anti-Ro but not anti-dsDNA in their glomerular eluates (Maddison and Reichlin 1979) and there is some literature correlating anti-C1q antibodies with renal disease (reviewed in Berden 1997). These reports indicate that anti-dsDNA antibodies are not the only nephritogenic mechanism in SLE and are therefore not completely synonymous with the development of lupus nephritis. It is also possible that a small number of patients with SLE develop a glomerulonephritis that is unrelated to their SLE.
Conclusion: Do anti-dsDNA antibody titres correlate with disease activity?

Although still quite controversial, it is thought that rising anti-dsDNA titres increase the risk of disease exacerbation in the following 8-10 weeks by a factor of two to three (data reviewed in Hahn 1998). The observation that anti-dsDNA antibody titre correlates with vasculitis, (Esdaile et al. 1996; Petri et al. 1991) may better explain the association between anti-dsDNA titre and disease activity. However, most work has been done on the role of anti-dsDNA antibodies in lupus nephritis, probably due to the technical difficulties in evaluating generalised vascular damage.

1.4 Organ binding and nephritogenicity of anti-dsDNA antibodies

There are a number of theories that attempt to explain the renal and vascular predilection of anti-dsDNA antibodies, these are discussed in detail elsewhere (Koffler et al. 1971; Schmiedeke et al. 1989; Kramers et al. 1994; Berden 1997 Madaio 1999). The study of anti-dsDNA antibodies as pathogenic entities hinge on the existence of proven cellular and glomerular toxicity. These have been studied both on a cellular and whole-animal level, and some of this data will be summarised below.

1.4.1 Binding of anti-dsDNA antibodies to cells

It has been shown that a subset of anti-dsDNA antibodies can penetrate living cells. Cell surface myosin I was shown to facilitate the entry of these antibodies not only
into the cell, but also into the nucleus. This mechanism allows the antibodies to gain close proximity to intracellular DNAase 1 (Madaio 1999). The activity of DNAase 1 has been shown to be inhibitable with anti-dsDNA antibodies (Emlen and Burdick 1988) and in some cell types DNAase 1 has a primary role in inducing apoptosis (Catchpoole et al. 1995). This may explain why some anti-dsDNA antibodies have been shown to be capable of effecting cell cycling (Madaio 1999).

In addition, there is extensive literature regarding cells of the lymphoid lineage expressing DNA (Bennet et al. 1985, Bennet et al. 1986) or a DNA like molecule on their surface, which in turn allows the binding of anti-dsDNA antibodies (Bennet et al. 1992). It has also been shown that when anti-dsDNA antibodies bind to mononuclear cells they induce cytokine production (IL-1, IL-6, IL-8, IL-10 and TNF alpha) (Sun et al 2000).

1.4.2 Binding of anti-dsDNA antibodies to the kidney causes glomerulonephritis

It has been suggested that antibody deposits act as a nidus for complement activation and therefore tissue damage (Cameron 1996). However, mice without C1q or C3 have developed lupus-like nephritis, suggesting that complement may not be required to mediate renal injury (Pickering et al. 2000)

Between them Ehrenstein et al. (1995) and Ravirajan et al. (1998) tested seven human anti-dsDNA monoclonals by implanting the cells in the peritoneal cavity of severe combined immunodeficient (SCID) mice. SCID mice were used because a normal mouse would mount an immune response against the monoclonal
cells and human antibody. Four of the seven monoclonals bound to the kidney and caused proteinuria. The antibodies that bound to the kidney did cause proteinuria and there was no proteinuria in the mice with no glomerular antibody deposits. The fact that only some of the anti-dsDNA antibodies caused proteinuria tallies with the suggestion that only some anti-dsDNA antibodies are nephrotoxic. No changes in renal architecture were found under light microscopy, although changes under electron microscopy compatible with SLE nephritis (notably glomerular basement membrane thickening and fusion of the glomerular foot processes) were detected in those mice in which the RH14 monoclonal antibody had been produced.

In summary, it seems highly probable that some, though not all, anti-dsDNA antibodies are nephrotoxic. The factors which might predict the possible pathogenicity of anti-dsDNA antibodies are extensively discussed elsewhere (Suzuki et al. 1993, Harada et al. 1994, Okamura et al. 1993).

1.5 Control of normal antibody production

This thesis will concern itself with the modulation of *in vitro* anti-dsDNA antibody production from SLE peripheral blood mononuclear cells (PBMC). It is therefore useful to outline the normal control of antibody production.

The activation of resting B-cells to become either antibody secreting plasma cells or long-lived memory B-cells can take one of two paths; T cell dependent or T-cell independent. If involved in the antibody response, helper T-cells will signal isotype switching and somatic hypermutation. Genetic analysis of monoclonal anti-dsDNA antibodies suggests that they have been through somatic hypermutation and have therefore received T-cell help (Gilkeson et al. 1995). Indeed, Voll et al (1997)
and Datta et al. (1997) have shown the existence of nucleosome specific T-cells in SLE patients. This discussion will therefore be restricted to the T-dependent pathway.

To receive T-cell help a B-cell must bind to the same pathogen as that T-cell. If a B-cell binds antigen by its surface immunoglobulin (sIg) it will ingest and present it on a MHC class II molecule. If the T-cell recognises the antigen with its T-cell receptor (TCR) and receives appropriate co-stimulation it will activate the B-cell. These activated B-cells (centroblasts) are classically CD38+ and CD20+. Centroblasts become part of a germinal centre within the lymphoreticular system, going through successive rounds of proliferation and somatic hypermutation during affinity maturation (MacLennan 1994). The centroblasts that compete successfully for antigen will move toward the periphery of the germinal centre and become centrocytes (Liu et al. 1989). These centrocytes will become antibody producing plasma cells (CD38+ CD20-) or memory B-cells (CD38-, CD20+) (Liu and Banchereau 1997). It is thought that memory cells do not need to enter the lymphoid system to proliferate and differentiate, being able to do so in the periphery (Liu et al. 1991). Where appropriate co-stimulation is not received, the T-cell may become a suppressor cell (see tolerance section 1.10).

1.5.1 Co-stimulatory molecules

1.5.1a CD28, CD80, CD86 and CTLA-4

A T-cell that binds an antigen with its T-cell receptor (TCR) must also receive co-stimulation from the APC if it is to respond positively and permit an immune response to this antigen. CD80 (B7-1) and CD86 (B7-2) are two such co-stimulatory
molecules. CD80 is expressed on dendritic cells and activated monocytes and B-cells, while CD86 is expressed at low levels on resting monocytes and B-cells. Both bind CD28 and impart positive co-stimulation. CD28 is constitutively expressed on all CD4+ T-cells and half of the CD8+ T-cells. After a T-cell is activated through CD28 it will express CTLA-4 on its surface. CTLA-4 has a higher affinity for CD80 and CD86 than CD28 and therefore competes out CD28 for their binding. CTLA-4 therefore acts to halt the immune response (June et al. 1994).

1.5.1b CD40

CD40-CD40L interactions have been shown to be obligatory for functional Th cell-B-cell interactions. CD40L is transiently expressed on activated T-cells and CD40 is constitutively expressed on APCs. CD40 signalling has been shown to be necessary for the growth, differentiation, resistance to apoptosis and effector functions of APCs (Datta et al. 1997). The role of CD40 is discussed in more detail in the following sections (section 1.5.2).

1.5.1c OX40-OX40 ligand

Like CD40 and CD40 ligand, OX40 and OX40 ligand are members of the TNF receptor and TNF families, respectively. It has been shown that OX40 is selectively expressed on activated T-cells, while OX40 ligand is expressed on activated B-cells, particularly those B-cells activated by CD40 or sIg cross linkage (Paterson et al. 1987, Calderhead et al. 1995). It is thought that OX40 directs B-cells to plasma cell
differentiation as opposed to proliferation or memory cell differentiation (Liu and Banchereau 1997).

1.5.2 Induction of the plasma cell / memory cell phenotype

A B-cell receives a mixture of signals, which control whether or not it proliferates, or differentiates into a plasma or memory B-cell. Research into this field is ongoing, however a number of different signalling molecules have been implicated in this sequence, namely CD40-CD40 ligand, OX40-OX40 ligand (both described above) and the cytokines interleukin-10 (IL-10), interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-6 (IL-6) (Liu and Banchereau 1997). [Cytokines are described in more detail in following sections].

To gain sensitivity to any of these signals a B-cell must first become activated. B-cells from healthy controls stimulated through CD40 ligation or cross linking of sIg become activated CD38+, CD20+ blast cells and proliferate; this process takes approximately 3 days (Arpin et al. 1995). The activated, “centroblast” B-cells will now express OX40 ligand and the IL-2 receptor as well as CD40, which is expressed regardless of activation state (Paterson et al. 1987, Calderhead et al. 1995, Jelinek and Lipsky 1988).

It has been found that if the “centroblasts” are stimulated for a further 4 days with CD40L, IL-10 and IL-2 they become CD38-, CD20+ memory B-cells that do not secrete antibody. However, if the CD40 stimulation is removed but the IL-10 and IL-2 is continued the “centroblasts” differentiate into CD38+, CD20- plasma cells that
secrete large quantities of antibody (Arpin et al. 1995). The relationship between CD40 ligation and antibody production has been a controversial issue. It was originally thought that CD40 ligation resulted in antibody production (Rousset et al. 1995). However, closer examination of this experimental system revealed that CD40 was inducing proliferation. After some time (which was dependent on the experimental conditions) the ratio of CD40 antibody to B-cells decreased to the point where enough B-cells were not receiving sufficient CD40 stimulation, this resulted in plasma cell differentiation (Liu and Banchereau 1997). OX40 stimulation has been found to promote plasma cell differentiation, regardless of the cytokines present (Stuber and Strober 1996).

After activation through CD40 or sIg, IL-2 is crucial in many experimental systems for blocking proliferation in favour of differentiation to either memory or plasma cells (Schilizzi et al. 1997). It appears that IL-10 and IL-2 work synergistically, IL-10 upregulating the expression of IL-2 receptor on B-cells (Fluckiger et al. 1993) as well as being a potent maturation/growth factor in its own right (Rousset et al. 1992). However, in most experimental systems IL-10 appears to optimise the differentiation signal given by other molecules, rather than commit B-cells to a specific fate itself (Schilizzi et al. 1997).

IL-4 appears to augment the proliferative response of B-cells already activated through CD40 or sIg, blocking their maturation to plasma or memory cells (Rousset et al. 1995). IL-6 appears to be important in maintaining plasma cells and promoting their differentiation (Roldan and Brieva 1991).
It appears that cytokines do effect the differentiation of B-cells; though slg, CD40 and OX40 have a greater effect on the direction of differentiation (be it to plasma or memory cell).

1.6 Apoptosis in SLE

Of clear interest in the study of any immune response is the responsible antigen. Its presence can alter the antibody response and the effect of cytokines. The antigen responsible for the anti-dsDNA antibody response in SLE has not yet been identified. However, it seems likely that the response will interact with any host DNA that is available. The possible sources of that DNA will now be discussed.

Two types of cell death have thus far been described in biological systems, necrosis and apoptosis. Necrosis occurs in response to toxic insult, while apoptosis is a biochemically-controlled sequence of events leading to cell termination (Majno and Joris 1995). Briefly, cells signalled to go into apoptosis complete a number of steps that are well-conserved across cell types. The cells shrink, the nucleus collapses and the plasma membrane undergoes anionic phospholipid exposure. Cells will compartmentalise their contents into membrane bound “apoptotic blebs” on their surface. These blebs have been shown to express many of the autoantigens that are targeted by the lupus-affected immune system on their surface (Levine and Koh 1999). Eventually these blebs disassociate into apoptotic fragments, which can be rapidly phagocytosed. A key difference between these two cytotoxic processes is that the clearance of necrotic cells incurs inflammation as the cell contents are spilled into
the surrounding tissues, whilst apoptotic fragments induce anti-inflammatory
cytokines (Herrmann et al. 1997) and are therefore normally cleared without
activating the immune system. Clearance of apoptotic fragments is so efficient that it
is not normal to find any apoptotic cells in histological sections (Levine and Koh
1999).

SLE patients have a higher than normal level of apoptotic fragments in their
blood (Amoura et al. 1997; Perniok et al. 1998). However, it is as yet uncertain
whether this is a reflection of the greater immune activation manifest in SLE, a
primary abnormality in the apoptosis signalling mechanism or a decreased clearance
of apoptotic cells.

Emlen et al. (1994) measured the rate of apoptosis in lymphocyte cultures
from patients with SLE, rheumatoid arthritis and healthy controls. The accumulation
of apoptotic cells was 2.4 times more rapid amongst SLE lymphocytes than either of
the other two groups. It was suggested that this reflected an abnormally high
apoptotic rate in SLE lymphocytes.

Fas (CD95) is the most well known pro-apoptotic signal and has been
implicated in the activation induced cell death of immune cells (Levine and Koh
1999). However, the fas gene and fas signalling appear to be normal in patients with
SLE (Wu et al. 1996; Lorenz et al. 1997). The increased levels of serum and cellular
fas (Bijl et al. 1998; Kovacs et al. 1997) correlate with the increased immune
activation present in SLE (Lorenz ML et al. 1997). It therefore appears that there is a
greater rate of apoptosis in SLE secondary to increased immune activation.
Herrmann *et al.* (1998) tested the ability of monocytes from healthy controls and patients with SLE to clear apoptotic cells *in vitro*. It was reported that SLE monocytes cleared apoptotic cells slower than monocytes from healthy controls, and that this abnormality was not dependent on the source of the dead cells. Therefore, it appears that the build-up in apoptotic material is due to the combination of an increased production and decreased clearance rate.

### 1.7 Cytokines

#### 1.7.1 Introduction to cytokines

As described above, the titre of anti-dsDNA antibodies mirrors disease activity in some patients. Clearly there is an immune signal that causes this variation in anti-dsDNA antibody titre. This same signal may cause a flare in disease activity, perhaps through an amplification of anti-dsDNA antibody production. Broadly speaking, the immune system has two signalling methods. Immune cells can bind directly to one another and transmit a message through cell-cell interaction or they can release a mediator that can diffuse throughout the body and pass a signal over a larger area; cytokines are such mediators.

Cytokines encompass a large variety of chemical mediators that are subdivided into interferons, interleukins and growth factors. Cytokines are low molecular weight proteins that are secreted by a variety of different cell types (Janeway 1997). Cytokines are involved in a wide variety of actions from initiating
and resolving both cellular and humoral immune responses to modulating the proliferation, differentiation and death of many different tissues.

Cytokines will bind to specific receptors on target cells. Receptors transduce this signal into a cell where it will be incorporated with the myriad of other signals that the cell is receiving. A cell may therefore respond differently to a cytokine depending on its context.

Cytokines can affect the same cell from which they are released. In this case they are said to exert an autocrine action. Alternatively a cytokine may be released from one cell and exert an effect on another cell close by, in this case it is said to be having a paracrine action. Rarely, a cytokine may be released into the circulation; this will enable that cytokine to affect cells throughout the body; this is called an endocrine action.

1.7.2 Types of cytokine: The Th1/Th2 paradigm

As research in T-cell immunology has developed it has become apparent that murine helper T-cell clones can be split into two functional groups. T-helper 1 (Th1) cells help polyclonal B-cell responses and delayed type hypersensitivity, inhibit Th2 cells and are associated with inflammatory actions. Th2 cells help polyclonal and antigen specific B-cell responses and inhibit the activation of Th1 cells; these are often called anti-inflammatory actions (Janeway 1997). These cells can be distinguished by the cytokines that they produce. Th1 cells produce IFN\(\gamma\) and IL-2, while Th2 produce IL-4 and IL-5.
Human T-cell clones often do not appear to adhere to this subgrouping, making it difficult to extend the paradigm into humans (Quint and Bolton 1989). Indeed, as more has been learnt about the actions of the different cytokines, it has become increasingly apparent that many cytokines have both Th1 and Th2-type, or pro and anti-inflammatory actions. Despite these reservations, the Th1/Th2 paradigm does encourage the visualisation of cytokines as part of a system rather than as a number of disparate signals.

Since its initial conception Th0 and Th3 cells have been added to the Th1/Th2 paradigm. Th0 cells are T-helper cells that are capable of producing both Th1 and Th2-type cytokines, most human T-cell clones fall into this category, whereas Th3 cells are thought to serve a suppressor/regulatory type function. On antigen specific stimulation Th3 cells produce IL-10 and TGF-beta which prevent an immune response to that antigen, this failure to respond to an antigen is called tolerance. (This will be described later in section 1.10).

1.8 Cytokines in SLE

1.8.1 Possible disease progression in SLE

If it is accepted that autoantibodies, such as anti-dsDNA, are responsible for the tissue injury seen in SLE, then three phases might be expected in the development of this disease. Firstly, there is a break in tolerance allowing the initial and continued production of pathogenic autoantibodies. Secondly, periods of immune activation that result in the continued production of these pathogenic autoantibodies. Thirdly, there
would be inflammation and concomitant tissue injury in the target organs resulting in
damage. As yet it is impossible to predict which subjects will break immune tolerance
or when they will do so, therefore this first phase of the disease cannot be reliably
studied over a set period of time. Consequently, this thesis will be confined to
measuring the effects of cytokines on existing anti-dsDNA responses.

1.8.2 The involvement of cytokines in disease progression

If a cytokine is driving the anti-dsDNA antibody response and that antibody is
responsible for disease flare, it seems likely that the level of that cytokine might have
some chronological relationship with disease exacerbation and serum anti-dsDNA
antibody titre. However, it also seems likely that the second, antibody production
phase and the third, tissue injury phase of SLE might run concurrently; thus cytokines
involved with both processes might be coincidentally elevated. In addition the
existence of a correlation between the level of a cytokine and disease activity only
implies an association with disease course, functional studies are required to show if
that association is causative, consequential or a confounding variable. It is also
possible that the changes in cytokine level that are relevant to disease progression
happen so quickly in vivo that they will be missed altogether by such studies;
meaning these studies present a false association. These considerations must be borne
in mind when examining this literature. Table 1.4 shows the correlations found
between serum cytokine level and disease activity. As it is being proposed that anti-
dsDNA antibodies are causally involved in disease pathogenesis, correlations
between serum anti-dsDNA titre and serum cytokine level are also presented (Table 1.5)

### Table 1.4 Correlations between serum cytokine levels and disease activity

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Number of patients tested</th>
<th>Correlation of serum level with disease activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>39 C/S</td>
<td>None with SLEDAI</td>
<td>Tokano et al. 1999</td>
</tr>
<tr>
<td>TNFα</td>
<td>9 L (2 years)</td>
<td>+ve with ECLAM</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td></td>
<td>20 C/S</td>
<td>+ve with SLEDAI</td>
<td>Park et al. 1999</td>
</tr>
<tr>
<td>sTNFRI (p55 and p75)</td>
<td>9 L (2 years)</td>
<td>+ve with ECLAM</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td></td>
<td>40 C/S</td>
<td>+ve with ECLAM</td>
<td>Davas et al. 1999</td>
</tr>
<tr>
<td>IL-1</td>
<td>20 C/S</td>
<td>None with SLEDAI</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td></td>
<td>15 C/S</td>
<td>None with SLEDAI</td>
<td>Chang 1997</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>20 C/S</td>
<td>None with SLEDAI</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td></td>
<td>15 C/S</td>
<td>None with SLEDAI</td>
<td>Chang 1997</td>
</tr>
<tr>
<td>IL-2</td>
<td>30 L (2 years)</td>
<td>+ve with SLAM</td>
<td>Cuadrado et al. 1993</td>
</tr>
<tr>
<td>IL-4</td>
<td>30 L (2 years)</td>
<td>None with SLAM</td>
<td>Cuadrado et al. 1993</td>
</tr>
<tr>
<td>IL-6</td>
<td>56 C/S</td>
<td>None with SLAM</td>
<td>Peterson et al. 1996</td>
</tr>
<tr>
<td></td>
<td>9 L (2 years)</td>
<td>None with ECLAM</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td>IL-10</td>
<td>72 C/S</td>
<td>+ve with SLEDAI</td>
<td>Houssiau et al. 1995</td>
</tr>
<tr>
<td></td>
<td>41 L</td>
<td>+ve with SLEDAI</td>
<td>Park et al. 1998</td>
</tr>
<tr>
<td>IL-15</td>
<td>20 C/S</td>
<td>None with SLEDAI</td>
<td>Park et al. 1999</td>
</tr>
</tbody>
</table>

**Key for Table 1.4 and 1.5**

C/S = cross sectional study  
L = longitudinal study  
+ve = statistically significant positive correlation (p < 0.05)  
-ve = statistically significant negative correlation (p < 0.05)

**Tables 1.4 and 1.5** Each table shows the number of patients tested and whether they were tested in a longitudinal or cross-sectional study. The correlation is shown and which disease activity score was used, see section 1.1.3. The reference is included on the same line as the study is described. All studies shown here used ELISA to measure cytokine levels, see following text.
Table 1.5 Correlations between serum cytokine levels and serum anti-dsDNA antibody titre

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Number of patients tested</th>
<th>Correlation of serum level with serum anti-dsDNA antibodies</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α</td>
<td>9 L (2 years)</td>
<td>+ve</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td>sTNF (p55 and p75)</td>
<td>40 C/S</td>
<td>+ve</td>
<td>Davas et al. 1999</td>
</tr>
<tr>
<td></td>
<td>9 L (2 years)</td>
<td>+ve</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td>IL-1</td>
<td>20 C/S</td>
<td>None</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>20 C/S</td>
<td>None</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td>IL-6</td>
<td>9 L (2 years)</td>
<td>+ve</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
<tr>
<td>IL-10</td>
<td>72 C/S</td>
<td>+ve</td>
<td>Davas et al. 1999</td>
</tr>
<tr>
<td></td>
<td>41 L</td>
<td>none</td>
<td>Park et al. 1998</td>
</tr>
<tr>
<td>IL-15</td>
<td>20 C/S</td>
<td>None</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
</tbody>
</table>

Table 1.5 Key and legend for Table 1.5 is the same as Table 1.4, above.

All the studies presented here used an ELISA to detect the levels of cytokines. Other studies have used a bioassay to detect cytokine levels. This method relies on measuring the effect of the serum on a cytokine sensitive cell line, however SLE serum has been shown to be cytotoxic (Klint et al. 2000) therefore studies that utilise this method have not been included here.

Some cytokines have not been analysed; for example IL-5 can effect antibody production (Arpin et al. 1995) yet no publications could be found that use a clinical measure to associate the level of IL-5 with disease state in patients with SLE.

There are publications that discuss the level of cytokines in SLE and compare them to that of healthy controls (HC) and other diseases. The findings of these papers are summarised in Table 1.6. However, it must be remembered that the existence of an autoimmune disease strongly implies the existence of a disordered immune system.
and therefore aberrant cytokine levels must be expected (and can be seen in table 1.6).

The serum level of a cytokine may be different in a patient with SLE as compared to a HC. However, it is difficult to ascertain whether this is a cause or a consequence of the disease process in SLE. There is further complication when SLE is compared with other autoimmune diseases which will produce abnormal cytokine levels for, presumably, different reasons. However, to put the changes in cytokine level described in tables 1.4 and 1.5 in context it is useful to know how those levels compare to those levels found in a HC. Table 1.6 will only summarise the serum levels of cytokines.

### Table 1.6 Serum level of cytokines in SLE as compared to healthy controls

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>SLE</th>
<th>Healthy controls</th>
<th>Serum level in SLE patients compared to HC</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>39 C/S</td>
<td>12 C/S</td>
<td>↑</td>
<td>Tokano et al 1999</td>
</tr>
<tr>
<td>TNF alpha</td>
<td>53 C/S</td>
<td>140 C/S</td>
<td>↑</td>
<td>Aderka et al. 1993</td>
</tr>
<tr>
<td>sTNFR (p55 and p75)</td>
<td>53 C/S</td>
<td>140 C/S</td>
<td>↑</td>
<td>Aderka et al. 1993</td>
</tr>
<tr>
<td>IL-1</td>
<td>20 C/S</td>
<td>13 C/S</td>
<td>↑</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>20 C/S</td>
<td>13 C/S</td>
<td>↑</td>
<td>Sturfelt et al. 1997</td>
</tr>
<tr>
<td>IL-2</td>
<td>30 L (2 years)</td>
<td>10 C/S</td>
<td>↑</td>
<td>Cuadrado et al. 1993</td>
</tr>
<tr>
<td>IL-4</td>
<td>30 (2 years)</td>
<td>10 C/S</td>
<td>↑</td>
<td>Cuadrado et al. 1993</td>
</tr>
<tr>
<td>IL-6</td>
<td>56 C/S</td>
<td>32 C/S</td>
<td>↑</td>
<td>Peterson et al. 1996</td>
</tr>
<tr>
<td>IL-10</td>
<td>72 C/S</td>
<td>30 C/S</td>
<td>↑</td>
<td>Houssiau et al. 1995</td>
</tr>
<tr>
<td>IL-12</td>
<td>39 C/S</td>
<td>12 C/S</td>
<td>↑</td>
<td>Tokano et al. 1999</td>
</tr>
<tr>
<td>IL-15</td>
<td>20 C/S</td>
<td>10 C/S</td>
<td>↑</td>
<td>Studnicka-Benke et al. 1996</td>
</tr>
</tbody>
</table>

**Key and legend for table 1.6**

Key and legend is the same as described in table 1.4 and 1.5 with the following additions.

↑ = Statistically significant higher concentration (p < 0.05)

↓ = Statistically significant lower concentration (p < 0.05)

Many studies have tested the *in vitro* cytokine production, however it has been found that this rarely reflects the serum value. This observation is thought to be due to
negative feedback effects on cultured cells from prolonged exposure to abnormal concentrations of cytokines *in vivo.* (Honda and Linker-Israeli 2000). It therefore seems likely that *in vitro* production is a response to disease activation as opposed to being a reflection of disease activation.

In most cases numerous studies exist that tested the same cytokine, where this is the case those studies that tested the highest number of patients or the longitudinal studies are included. There was generally good agreement between the different studies.

**1.8.3 Why study IL-10?**

It is unlikely that sufficient cells will be obtained to test every cytokine of interest, therefore only one or two could in practice be selected. The best way to select a cytokine that might be involved in disease flare would be a longitudinal study. Such a study would follow the cytokine levels of a cohort of patients over time. The cytokines that altered just prior to an exacerbation in disease, or an increase in anti-dsDNA titre, would be the most likely to be involved in a disease flare. However, this study would not preclude the possibility that the change is a consequence as opposed to a cause of the SLE flare. Existing studies have demonstrated that TNF alpha and IL-10 satisfy these criteria.

**1.8.3a TNF alpha**

TNF alpha was not originally selected for two reasons. Firstly, it is thought likely that TNF alpha is involved in the damage associated inflammation of lupus flare (reviewed by Kelley and Wuthrich 1999) so is likely to be elevated as a consequence
of SLE flare rather than a cause. Secondly and most importantly, previous studies have demonstrated that it has no role in elevating the *in vitro* IgG production of lupus PBMC (Linker-Israeli *et al.* 1991). While this may not reflect its role in IgG anti-dsDNA antibody production, IL-10 has been implicated in the elevated IgG production seen in SLE (see below). This finding has raised the expectancy that IL-10 is causally involved in anti-dsDNA antibody production.

1.8.3b IL-10 and antibody responses in healthy controls

It has been found that IL-10 can effect antibody production through its effect on B-cell differentiation. This effect has been described in section 1.5.2. However, it is thought that B-cells need to be pre-activated in order to become sensitive to IL-10. Unlike patients with SLE, HC do not normally have appreciable numbers of activated B-cells in their periphery (Spronk *et al.* 1996). Therefore IL-10 alone would not be expected to have a significant effect on antibody production in HC and indeed this was found to be the case (Llorente *et al.* 1995).

1.8.3c The effect of IL-10 on the response to antibodies in healthy controls

IL-10 increases the surface expression of FcγR on monocytes (te Velde *et al.* 1992). Therefore IL-10 might contribute to antibody dependent cytotoxicity, by increasing both IgG antibody production and expression of the relevant receptor (Stordeur and Goldman 1998).
1.8.3d The effect of IL-10 on the antibody production in diseases other than SLE

The effect of IL-10 on in vitro antibody production has not been widely studied in diseases other than SLE. IL-10 has been shown to increase the anti-collagen antibody production from pure B-cell cultures from patients with type I diabetes (Stein et al. 1997).

1.9 IL-10 in human SLE

1.9.1 IL-10 and antibody production in human SLE

Llorente et al. (1995) have tested 17 patients with a wide range of disease activities and demonstrated that IL-10 caused a significant increase (350%) in total IgG antibody production in T-cell depleted cultures while IL-6 had no effect. Serum IL-6 levels have been shown to correlate positively with serum dsDNA antibody titre in some studies (see Table 1.6). These results suggest that despite the high levels of IL-10 in SLE, its signal has not reached saturation point. Saturation appears to have affected the IL-6 signal, as anti-IL-6 decreases in vitro IgG production while IL-6 addition makes no difference (Nagafuchi et al. 1993).

1.9.2 IL-10 and antibody class switching

The majority of the pathogenic anti-dsDNA antibodies have been found to be IgG1 or IgG3. IL-10 is capable of inducing switching to these subtypes in cultures of CD40 activated, naive, human B-cells (Briere et al. 1994). This may be a confounding observation in SLE, as IL-10 has been shown to be high in lupus flares and therefore may induce class switching in the pathogenic antibodies without actually being responsible for their amplification.
1.9.3 IL-10 and the female bias of SLE

Papers seeking to explain the female bias of SLE have demonstrated that oestrogen can worsen murine lupus while testosterone can ameliorate it (Roubinian et al. 1979, Carlsten et al. 1990). Kanda et al. (1999) demonstrated that oestrogen can increase *in vitro* anti-dsDNA antibody production from lupus PBMC, an effect that co-administration of anti-IL-10 partially (50%) blocked; while the other anti-cytokine antibodies tested had no effect (anti-TNF alpha, anti-IL-1, anti-IL-2, anti-IL-4 and anti-IL-6).

1.9.4 IL-10 and apoptosis

In section 1.6 it was suggested that there may-be some quantitative abnormalities in apoptosis in patients with SLE. IL-10 has been shown to cause a significant (20% in 5 day cultures) increase in apoptotic rate amongst T-cells from patients with active but not inactive disease (Georgescue et al. 1997). Moreover, this increase could be blocked (by 50%) by addition of anti-fas; thus suggesting that IL-10 was increasing apoptosis as opposed to decreasing clearance. If IL-10 can increase the antibody production and the apoptotic rate (or decrease clearance, which has not been tested) then IL-10 could be central in the causation of SLE flare by increasing both anti-dsDNA antibody titres and antigen through increased apoptotic rates. However, as yet the *in vitro* effect of IL-10 on anti-dsDNA antibody production in human cells has not been described.
1.10 Peripheral tolerance and the Th subtype of the anti-dsDNA response

1.10.1 IL-10 and peripheral tolerance

Contrary to the suspected role of IL-10 in causing SLE, IL-10 has been shown to be central in the positive feedback loop that induces and maintains peripheral tolerance (the Th3 subtype). This topic is of interest to anyone studying autoimmune disease, so will be summarised here.

1.10.2 The inhibitory effects of IL-10 on inflammation

On receiving a pro-inflammatory stimulus (for example lipopolysaccharide), granulocytes will become activated. This will incur the production and release of pro-inflammatory cytokines such as TNF alpha, IL-1, IL-6 and IL-8 which will further potentiate the activation of these cells (De Waal Malefyt et al. 1991). The physiological purpose of granulocyte activation is pathogen destruction. This is effected by granulocyte degranulation, releasing cytotoxic free-radicals and nitric oxide (NO). IL-10 will inhibit the above pro-inflammatory cytokine release from monocyte/macrophages (De Waal Malefyt et al. 1991; Bogdan et al. 1991), neutrophils (Cassatella et al. 1993), eosinophils (Takanaski et al. 1994) and mast cells (Arock et al. 1996) and the degranulation of macrophages (Bogdan et al. 1991).

Moreover, IL-10 enhances the in vitro production of the IL-1 receptor antagonist by monocytes (Jenkins et al. 1994) and neutrophils (Cassatella et al. 1994). Approximately 7 hours after this initial production of TNF alpha and the other pro-
inflammatory cytokines, IL-10 will be released, which will stop the inflammation and eventually block its own release (Moore et al. 1993). In conclusion: IL-10 is a late product of inflammation that acts to resolve that inflammation regardless of what cell is responsible.

1.10.3 Models of peripheral tolerance

Peripheral tolerance has been examined in relation to many forms of allergy with a view to treatment. Specific immunotherapy (SIT) consists of administering small repeated doses of the peptide responsible for the allergic response. SIT has proved particularly successful in the treatment of bee venom allergy. The immunodominant epitope in bee venom is phospholipase A2 (PLA$_2$). Patients successfully treated with SIT lose the in vitro proliferative responses of their PBMC to PLA$_2$. However, anti-IL-10 treatment, or the addition of pro-Th1 (IL-2 or IL-15) or Th2 (IL-4, IL-5 or IL-13) cytokines reconstitutes the proliferation and cytokine production of PBMC cultures (Akdis and Blaser 1998).

1.10.4 Mechanism of peripheral tolerance

As was mentioned previously (section 1.7.2), Th3 cells are thought to be suppressor cells, involved in antigen specific tolerance. APCs and the effect of IL-10 on them is thought to be central in the induction of tolerance. IL-10 down-regulates the expression of MHC class II molecules (de Waal Malefyt et al. 1991), CD80 and CD86 (Willems et al. 1994) on monocyte/macrophages. This effect blocks the
necessary co-stimulation for effective antigen presentation and may result in the
induction of a Th3 phenotype in the T-cell specific to that antigen (Akdis and Blaser 1998). Th3 cells produce IL-10 on further antigen stimulation, therefore producing a positive feedback loop. The consequent high levels of IL-10 block the inflammatory cascade, protecting the antigen from an immune response. As with Th1 and Th2 cells, active Th3 cells produce IL-2 as a growth factor (Akdis and Blaser 1998).

1.10.5 What Th subtype is the anti-DNA response?

It is widely believed that the pathogenic response in SLE is Th2 (Barcellini et al. 1996). However, both Th1 and Th2 cytokines are raised in SLE, see table 1.7. The IL-10: IFNγ ratio has been found to increase during disease flare (Hagiwara et al. 1996), but IL-10 can act as a Th3 type cytokine not just as a Th2, as is often assumed (Barcellini et al. 1996).

Voll et al. (1997) made anti-nucleosome specific T-cell clones from one HC, one newly diagnosed SLE patient and one patient with long standing SLE. Three clones each from the HC and the newly diagnosed SLE patient and one from the long-standing SLE patient induced anti-dsDNA antibody production from autologous B-cells. The amounts of IL-4, IL-10, IFNγ and IL-2 produced from each clone were quantitated. All the anti-nucleosome T-cells were Th0 or Th1, though Th2 clones that were not nucleosome specific were made with this protocol. If the clones were ranked by the quantity of each cytokine that they produced and by the amount of anti-dsDNA antibody that they induced these are the correlations by rank that would be found. In the HC’s clones IL-2, IL-4 and IL-10 correlated positively with anti-dsDNA antibody
production, while there was a negative correlation with IFNγ. In the clones from the newly diagnosed SLE patient, there was a positive correlation between anti-dsDNA induction and IFNγ and a negative correlation with IL-10. All the anti-nucleosome specific clones from the long-standing SLE patient failed to produce detectable levels of IL-10, whereas nearly all the clones from the other two individuals did produce detectable levels. This evidence is at best anecdotal due to the very small numbers involved. However, it is compatible with the role of IL-10 in tolerance induction and suggests that the pathogenic response to nucleosomes is a Th1, IFNγ-dependent, response. The existence of anti-nucleosome T-cells in the HC implicates a deficit in peripheral tolerance in the pathogenesis of SLE.

1.10.6 Could IL-12 induce the Th1 anti-nucleosome response?

IL-12 is considered necessary to induce a Th1 phenotype (Gately et al. 1998). In vitro studies in humans and in vitro and in vivo studies in mice have shown that IL-12 promotes Th1 type responses (Mannett et al. 1993; Marshall et al. 1995; Hsieh et al. 1993 and Heinzel et al. 1993). IL-12 induces a Th1 type phenotype through three interactions with IFNγ. Firstly, on initial antigen encounter it signals naive T-cells to differentiate into a population of Th1 cells which will secrete IFNγ on activation (Hsieh et al. 1993). Secondly, it is a necessary co-stimulus for maximal IFNγ secretion by differentiated Th1 cells (DeKruyff et al. 1995). Thirdly, it promotes the development of IFNγ producing Th1 cells from resting memory T-cells interacting with an antigen that they have encountered previously (DeKruyff et al. 1995).
Some models required IFNγ for IL-12 to induce a Th1 type response. However, IFNγ alone is not enough to substitute for IL-12 (Gately et al. 1998). It has been suggested that IFNγ is a necessary co-factor for IL-12 to reach maximal secretion (Dighe et al. 1995).

Although it is usual for IL-12 to promote Th1 responses it has been found that in certain conditions it will promote a Th2 type response (Caspi et al. 1998). For example, co-stimulating short T-cell lines from HC with IL-12, certain antigens (for example tetanus toxoid) and anti-CD3 resulted in the production of IL-10 in large quantities (Windhagen et al. 1996). It has been suggested that this represents a negative feedback loop which prevents an inappropriately chronic autoimmune response from occurring (Gately et al. 1998), as IL-10 can inhibit the production of IFNγ and IL-12 (D’Andrea et al. 1993).

1.10.7 Could IL-12 reverse tolerance?

1.10.7a IL-12 increases the expression of co-stimulatory molecules

Unlike IL-10, IL-12 is capable of enhancing the ability of bone marrow derived dendritic cells to activate T-cells. This is thought to be due to causing an increase in the surface expression of CD80 (Kelleher and Knight 1998). IL-12 has also been shown to up-regulate the expression of CD40 ligand on human T-cells. It therefore seems possible that IL-12 could break IL-10 induced tolerance by reversing the IL-10 induced down regulation of co-stimulatory molecules.
1.10.7b IL-12 can reconstitute immune responses

Unfortunately, the effect of IL-12 on the SIT model of peripheral tolerance has not been investigated in humans. However, it has been found that IL-12 can reverse IL-10 induced antigen specific non-responsiveness in mice to a variety of antigens (for example Leishmania and Candidiasis), by inducing a Th1 type response. However, IL-12 is unable to produce this effect in the presence of high quantities of IL-10 (Dighe et al. 1995 and Romani et al. 1994). This could be due to the fact that IL-10 would inhibit the further production of IL-12 and IFNγ, and blocks the expression of the signalling chain of the IL-12 receptor. (D’Andrea et al. 1993 and Mosmann et al 1994).

1.11 Induction signals for IL-12 and IL-10 that might be relevant to SLE

IL-12 can be induced by CD40L signalling (Gately et al. 1998) and there is an abnormally high expression of CD40L in patients with active SLE (Datta et al. 1997). This together with the observation that anti-nucleosome specific T-cell clones are of the Th1 type has lead to the suggestion that IL-12 is driving the T-cells responsible for anti-dsDNA antibody production in SLE (Datta 1998).

Antibody responses are driven by antigen; the putative antigen in SLE is the nucleosome. Sato et al. (1999) showed that DNA extracted from the circulating immune complexes of 9 patients with SLE induced IL-12 and IFNγ production from the monocytes of HC. Unfortunately, the measurement of no other cytokines were undertaken and the effect on monocytes from patients with lupus was not tested. As
mentioned previously, monocyte IL-10 production is induced in response to apoptotic fragments in HC; these studies have not been replicated in patients with SLE (Herrmann et al. 1997).

1.12 Are IL-10 levels too low in SLE? (Thus preventing tolerance induction)

It is possible that the raised IL-10 levels seen in active SLE are actually a failed effort to induce tolerance. Mongan et al (1997) stimulated B-cells and monocytes (with lipopolysaccharide) from HC and SLE patients and measured the consequent release of IL-6. The monocytes and B-cells from SLE patients required ten times the amount of IL-10 that the HC required to achieve the same level of IL-6 inhibition, however, this result has not been replicated by other authors (Linker-Israeli et al 1999).

1.13 IL-12 and antibody production

1.13.1 The effect of IL-12 on antibody production from healthy controls

If IL-12 is one of the causative factors in SLE, it might be expected to have a role in antibody production, though this role would be indirect, as B-cells do not express the signalling β2 IL-12 receptor chain. The role of IL-12 in antibody production has mostly been examined in the mouse. It was recently shown that IL-12 can enhance antigen specific IgG2a, IgG2b and IgG3 antibody production in protein immunised mice by as much as 1000-fold (Germann et al. 1995). These isotypes are regarded as the Th1 isotypes thus it is perhaps unsurprising that IL-12 induced an increase in their production. Indeed after primary immunisation with hen egg lysozyme, IL-12
suppressed IgG1 responses. However, after several boosts, IL-12 induced a modest (2-5-fold) enhancement of IgG1 production (Buchanan et al. 1995).

Most healthy subjects have a natural autoantibody repertoire that will contain IgM autoantibodies to thyroglobulin, ssDNA and other self antigens. CD5+ B-cells are thought to be the source of these autoantibodies (Pisetsky et al. 1990). Jones (1996) tested the in vitro production of IgG by cultures of pre-activated B-cells and CD4+ cells from human HC. It was found that CD5+ B-cells were preferentially activated (over CD5- B-cells) to produce IgG antibody by IL-12. IL-12 did not affect the growth of CD5+ B-cells or the expression of CD5. None of the other cytokines tested had a CD5+ specific effect (IL-1, IL-4, IL-5, IL-10, and TNF alpha and TGF beta were also tested).

1.13.2 IL-12 and antibody production in patients with SLE

Houssiau et al (1997) tested 14 patients with active disease and showed that IL-12 decreased the number of anti-DNA producing cells by 32% in 48-hour cultures (as measured by ELISPOT). This is surprising in the light of the existing data on IL-12. However, this finding could be of great therapeutic interest and therefore the mechanism responsible deserves further elucidation.
1.14 Summary

SLE is a chronic, incurable disease and as such should be the target of further research to improve on existing treatments. SLE presents with a wide variety of clinical symptoms, renal disease being one of the more common serious complications. Anti-dsDNA antibodies have been causally linked with lupus nephritis and disease flare.

If the immune signal responsible for anti-dsDNA antibody expansion is consistent and could be defined and blocked it might represent a novel treatment for SLE.

IL-10 and IL-12 have been studied, as it seemed possible that some interesting results might be gained. IL-10 has been extensively examined in SLE, but as yet its effect on human anti-dsDNA antibody production has not been described. It is generally agreed that IL-10 is centrally and causally associated with SLE. Contrary to these findings there is some evidence to suggest that IL-10 may be capable of inducing antigen specific tolerance.

IL-12 has not been as well studied in patients with SLE. The existing literature in SLE suggests that IL-12 might be therapeutic through inhibition of antibody production. However, the effects of IL-12 on murine and normal human B1-cell antibody production do not support this role. These results together with the finding that anti-nucleosome specific T-cells are of the Th1 phenotype suggests the role of IL-12 in SLE requires further research.
To study the effect of cytokines on anti-dsDNA antibody production a simple model has been proposed. The culture of total PBMC with or without cytokine and the consequent measure of IgG anti-dsDNA, ssDNA and total IgG antibody levels by ELISA.

Initial hypothesis

IL-10 is causally associated with SLE through its effect on anti-dsDNA antibody production; it will therefore increase in vitro anti-dsDNA antibody production. In SLE, IL-10 overproduction imbalances the immune system through its ability to inhibit IL-12 production. The addition of IL-12 will neutralise some of the effects of IL-10 and therefore inhibit in vitro anti-dsDNA antibody production.

General aim

To measure the effect of IL-10 and IL-12 on the in vitro anti-dsDNA antibody production of peripheral blood mononuclear cells from patients with systemic lupus erythematosus and determine how these cytokines have the effects that are observed.

Objective of chapter 4

Measure the effect, if any, of IL-10 and of IL-12 on the in vitro anti-dsDNA antibody production of PBMC taken from patients with SLE.
Objective of chapter 5
Measure the effect, if any, of IL-10 on the \textit{in vitro} anti-dsDNA antibody production of PBMC taken from patients with SLE that have been depleted of either monocytes or T-cells; and compare with the effect of IL-10 on total PBMC.

Objective of chapter 6
Measure the effect of IL-10 on the rate of T-cell apoptosis and test whether or not this effect varies with disease activity. Use statistical analysis to compare the predictive power of 'disease activity' with that of 'IL-10-induced T-cell apoptosis' to predict the effect of IL-10 on anti-dsDNA antibody production.
2.0 Methods

2.1 Cell separation

2.1.1 Separation of peripheral blood mononuclear cells (PBMC) from whole blood

Venous blood samples from patients with SLE were collected in sterile, heparinised tubes. The blood samples were prepared using sterile equipment and aseptic technique in a laminar flow hood (Gelaire) at room temperature. Before separation was started, a 1 millilitre (ml) portion of plasma was taken from each blood sample and placed in a 1 ml screw top container (Eppendorf). The plasma was spun for 5 minutes at 1300 rpm before being transferred to a second 1ml screw top container and stored at -20°C.

The blood samples were diluted 1:1, with RPMI (GibcoBRL), in 50ml tubes (Falcon). This suspension was taken in 25 ml samples and overlaid onto an equal volume of Histopaque 1077 (Sigma) in 50 ml tubes and then spun for 25 minutes at 2000 rpm; this was done at room temperature to optimise the separation. While the Histopaque layers were spinning, 5 ml aliquots of RPMI were placed into an appropriate number of fresh 50ml tubes. Once the layers had stopped spinning the peripheral blood mononuclear cells (PBMC) were immediately removed from the interface with a sterile Pasteur pipette (Alpha Laboratories) and placed in the 50 ml tubes that had received the 5ml aliquots. This method was used to minimise the time in which the cells were exposed to a high concentration of Histopaque, as it has
cytotoxic properties. To remove any remaining Histopaque, the cells were washed twice by the following procedure. The cell suspension was diluted at least 1:1 with RPMI in a 50ml tube and spun for 15mins at 1500rpm. The medium was then discarded, the cells resuspended to 50mls with RPMI and the suspension spun again for 15mins at 1500rpm. After this second wash the cells were diluted to a known volume in supplemented RPMI (supplemented with 1% Glutamine, 2% Non-essential amino acids, 0.2% Gentamycin, 1% Sodium Pyruvate and 1% Penicillin and streptomycin all from GibcoBRL and 10% heat inactivated fetal calf serum from Sigma). 10 ul of this suspension was diluted 1:10 with RPMI in a 1ml screw top container (Eppendorf) and then 10ul of this diluted suspension was added to an equal volume of ethidium bromide acridine orange solution in a second screw top container. 10ul of this ethidium bromide acridine orange/cell suspension mixture was introduced under the coverslip of a haemocytometer (Neubauer) and the number of viable (green) cells were counted with a UV light microscope, dead (orange) cells were ignored. Preliminary experiments demonstrated that >95% of cells were viable at this stage. After counting, the cells were diluted to 2 by 10^6 ml^-1 in supplemented RPMI and put up in 0.5 ml cultures in 12 by 75mm round bottomed culture tubes from Falcon. The incubator used for culture held the cells at 37°C in 5% CO₂.

2.1.2 T-cell and monocyte depletion from total PBMC

The combination of magnetic beads from Dynal (Dynabeads) and a specific antibody achieved the depletion of each of these cell types. Murine anti-human CD14 from Pharmingen was used to remove monocytes and murine anti-human CD3
(Pharmingen) was used to remove T-cells. Immediately after the PBMC had been removed from whole blood, they were cooled on ice. Throughout this procedure the cells and all the solutions brought into contact with the cells were kept sterile and at 4°C. This low temperature was maintained to prevent both antibody capping and to stop the antibodies from activating the cells.

A volume of the cell suspension that contained over double the number of cells that were required after depletion (to allow for non-specific loss) was transferred into a 12 by 75mm round bottomed tissue culture tube and spun for 10 minutes at 1500 rpm. The medium was then discarded and the antibodies were added at 1ug per million cells (antibody solutions were stored at 100ug/ml). Previous experiments using FACs analysis demonstrated that this concentration supersaturated the cells (see section 2.7.1). After addition of antibody, the cells were diluted to a volume of two ml with RPMI and incubated on a roller for 60 minutes at 4°C. This step allowed the antibodies to adhere to their target. A volume of 2mls was used to prevent a significant number of cells being lost on the wall of the container. The cells were then transferred to a 50ml tube (Falcon) and washed twice with 30mls of RPMI at 1500rpm for 10 minutes. This procedure was intended to wash off the excess unbound antibody. The cells were then placed in 15ml polypropylene tubes (Falcon) and diluted in RPMI to 2.5 by 10^6 cells per ml (assuming no cell loss since the initial count). Dynal states that this is the optimum cell concentration for depletion.

Dynabeads (with sheep anti-mouse IgG antibody attached to their surface) were washed twice. This was done by placing the required amount of Dynabeads in a 12 by 75mm tube, placing this tube on the magnet for 5 minutes and then removing the
solution in which the Dynabeads were suspended. The Dynabeads bound to the magnet were then reconstituted with an equal volume of ice-cold RPMI as was removed. This was done to remove the azide in which the Dynabeads were supplied. The washed Dynabeads were added to the cells such that there were four beads for every cell in the suspension, Dynal state that this is the optimum ratio for depletion. The suspension was then incubated on a roller at 4°C for 30 minutes; thus allowing the Dynabeads to adhere to the antibody coated cells.

The cells bound to beads were removed by placing the suspension on a magnet for 2 minutes. The suspension was then aspirated with a sterile Pasteur pipette. The aspirated suspension was then placed on the magnet for a second 2 minutes to ensure all the beads had been removed. Then the cells were pelleted by spinning at 1500rpm for 10 minutes, resuspended in an appropriate concentration and counted with acridine orange ethidium bromide solution (see section 2.2.1). Typically about 85% of the target cells were removed, see figs 2.1a-d. In some cultures the percentage of B-cells were quantitated (see 2.11.2), in this case cultures were put up to achieve the same concentration of B-cells in depleted cultures as in non-depleted cultures. In other experiments, cells were put up at 2 by 10^6 cells per ml in half ml cultures regardless of B-cell number. Both these culture conditions were utilised as two issues needed to be addressed. First, in order to compare the antibody production between depleted and non-depleted cultures, the number of B-cells must be equal. Second, this thesis aims to measure the effect of IL-10 on antibody production and many of these effects are imparted through cell-cell interactions; for example IL-10 can effect the expression of co-stimulatory cell-surface molecules (see section...
1.10.4). The number of interactions between cells will be effected by the concentration of cells in the suspension. Therefore, by maintaining the same concentration of cells, the effect of IL-10 on a similar number of interactions, but in the absence of T-cells or monocytes, can be tested. However, it must be remembered that this second approach only allows the comparison of antibody production between untreated and IL-10 treated cultures within a single patient, a single experiment and a single “type of depletion” (“Types of depletion” meaning: Non-depleted, monocyte depleted or T-cell depleted).

2.2 Cytokines

Both IL-10 and IL-12 were originally obtained from Sigma (IL-10 EC\textsubscript{50} = 2ng/ml; IL-12 EC\textsubscript{50} = 0.2ng/ml (less than 0.1ng/ug endotoxin). Stock solutions were stored at -80°C at 5ug/ml in 12ul aliquots. Prior to use the stock solutions were diluted in supplemented RPMI such that 10ul added to each cell culture would achieve the required concentration of cytokine. Once the cells had been dispensed in culture, cytokine was immediately added to all treated samples and 10ul of supplemented RPMI was added to the untreated cultures as a control.

IL-10 (from ImmunoKontact and R&D) and IL-12 (from R&D) were also used in the course of this project. Again the manufacturers tested for endotoxin level and EC50 (which was identical for all the different companies). In each case, cytokine treated cultures were administered with a volume of cytokine solution to obtain the required final concentration, and the untreated cultures were treated with
the same volume of the buffer (filtered by 32mm Acrodisc [Pall corporation] for sterility) in which the cytokine was diluted. The cytokines produced compatible results regardless of source, verifying the quality of each cytokine.

To assess the effect of IL-10 and of IL-12 on antibody production, PBMC from each patient were cultured in triplicate without cytokine or with IL-10 or IL-12, then the supernatants taken and tested by ELISA for antibody production (see section 2.4).

2.3 Supernatant harvesting

A fresh sterile Pasteur pipette was used to harvest each of the supernatants, which were then dispensed into 1ml screw-top containers (Eppendorf). These 1ml screw top containers were spun at 1300 rpm for 5 minutes to remove any cell debris, the resulting supernatants were then transferred into a further 1ml screw top container which was clearly labelled. Supernatants were either used immediately or stored at -20°C before being tested by ELISA. In either case, supernatants that were to be compared were treated identically and, wherever possible, tested on the same ELISA plate.

2.4 ELISA methodology

2.4.1 Double-stranded DNA antibody ELISA

This methodology was adapted from O’Dar et al. [1988] (Citrate buffer (0.015M sodium citrate, 0.15M sodium chloride) was filtered for sterility with 32mm
Acrodiscs from the Pall corporation. Double stranded DNA from Sigma was dissolved at 500μg/ml in this filtered citrate buffer. The DNA solution required two days at 4°C to dissolve fully. Maxisorb plates (NUNC) were divided vertically into two equal halves with a permanent marker pen. The outermost ring of wells were not used to avoid the "edge-effect". Both halves were coated at 50ul per well with either citrate buffer (uncoated) or the DNA solution (coated). The plates were then incubated for two hours at 37°C in a dry incubator. Prior to this and all other incubations the ELISA plates were wrapped tightly in Clingfilm.

The plates were washed twice by hand in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS Tween). The supernatants were added neat, and in duplicate, at 50ul per well. A standardised normal serum from Pru procurements and a positive control serum from an active patient were also added, in singular, to each plate. The serum samples were added at 50ul per well at a dilution of 1:100 with four more halving dilutions moving horizontally across the plate. All samples were added to both antigen-coated and antigen-uncoated sides of the plate. The plates were then incubated at 37°C for 90 minutes in a dry incubator.

The plates were washed four times, by hand in PBS Tween. Goat anti-human IgG alkaline phosphatase conjugate (Sigma) was dissolved at 1:500 in PBS Tween and added at 50ul per well to all used wells. The plates were then incubated overnight at 4°C in a refrigerator. The plates were washed four times, by hand in PBS Tween then twice in bicarbonate buffer (BIC). Substrate tablets from Sigma were dissolved
in BIC at 1mg/ml with 0.2% one molar MgCl₂. This solution was then added at 50ul per well to all used wells. The plates were then put in a dry incubator at 37°C. Optical densities (ODs) were read at 5, 30 and 60 minutes by comparing the densities at 405nms wavelength with that at 490nms wavelength on an ELISA plate reader (Dynatech).

2.4.2 Total IgG ELISA

The total IgG ELISA followed the same protocol as the DNA ELISA with the exception of the first two hours. A Fab₂ monoclonal mouse antibody against human IgG (Sigma) was used as the capture antibody. The capture antibody was diluted 1:1000 in BIC buffer and dispensed at 50ul per well on the antigen coated side, with BIC at 50ul per well on the antigen uncoated side. The plates were then incubated for 1 hour at 37°C before being washed twice in PBS. The plates were then blocked with 200ul per well of 2% casein (from Sigma) in PBS and incubated for 1 hour at 37°C. The plates were washed twice by hand in PBS Tween. The samples to be tested were added as described in the anti-dsDNA ELISA, but at a ten times greater dilution; supernatants at 1:10 and serum samples starting at 1:1000. The addition of conjugate and substrate was performed as described in the anti-dsDNA ELISA.

2.5 Data analysis

Three duplicate ODs were measured and averaged for each treatment in each patient. The Shapiro-Wilk test demonstrated that the antibody production was not normally distributed; therefore non-parametric tests were used. In initial experiments the
Kruskal Wallis test was used to ensure that there was no significant variation between the antibody production from identically treated cultures, which could be due to uneven distribution of B-cells.

The object of this study is to assess the difference in antibody production induced by cytokine. In all cases the raw data will be presented. However, to see the effect of cytokine optimally, the difference between untreated and cytokine-treated antibody production will be presented. This can be presented as raw difference, (Mean treated OD – Mean untreated OD) or as percentage difference.

\[
\text{Mean percentage change in OD} = \frac{(\text{Mean treated OD} - \text{Mean untreated OD}) \times 100}{\text{Mean untreated OD}}
\]

This formula combines two variables (variation in spontaneous antibody production and treatment condition). It allows for the large variation in spontaneous antibody production exhibited by patients with SLE by standardising for spontaneous antibody production. Both these computations focus on the change induced by cytokine, making it ideal for graphic representation of any relationships found with multiple regression analysis of the raw data. However mean percentage change must be used with care, as if there is no relationship between untreated and treated OD (it is proportional in this thesis), as untreated OD gets larger, the mean percentage change will tend to get smaller. This caveat becomes important if mean percentage change is correlated with any variable that correlates with untreated OD (this is not done in this thesis) as this could prove to be a confounding variable. Where the
relationship between untreated and treated OD is not close, difference in OD (Mean treated OD – Mean untreated OD) is used to focus the reader on the change induced by cytokine. It must be remembered when viewing such results that there is no standardisation for spontaneous OD and therefore an apparently large change may be small in relation to the ODs exhibited by the patient. Conversely, when using mean percentage change, relatively small changes may appear large if the spontaneous antibody production of the patient is small.

If percentage change, a linear scale, is to be computed from a logarithmic scale (OD) it is important to ensure that the ODs used are limited to the linear section of sensitivity of that ELISA. On each ELISA, a positive control was diluted across the plate. The ODs followed a linear descent with increasing dilution, until the lower limit of sensitivity of that ELISA was reached (usually approximately 0.15) at which point the descent flattened out. Only OD greater than the lowest point of linear descent were used in this study. This allowed a linear measure of change (percentage change) to be used. In addition, if ODs are to be compared as accurate measures of the level of antibody, the linear section must be used as any differences in OD outside this section is likely to be due to noise in the system.

The Mann-Whitney test was used to assess whether the cytokine-induced change in antibody production varied between HC and patients with SLE (SLE patients with active disease were compared separately from patients with inactive disease). To test whether or not the change in antibody production varied with disease activity these two parameters were correlated with the Spearman rank correlation.
In chapters 4, 5 and 6, individual factors that might be responsible for the variation in antibody production were assessed. In order to assess many factors simultaneously a multiple linear regression was used. This meant that the above formula was not required in the analysis of the data; however it was used as a convenient form to present the data graphically, if the aforementioned criteria were satisfied.

SPSS (statistics package for social scientists) was used to perform the statistical analysis, Microsoft Excel and SPSS were used to present the graphs and Microsoft Word was used to write the thesis and tables.

2.6 Immunofluorescence staining for FACS analysis - general methodology

The cells were stained in a 96 well, round bottomed tissue culture plate (NUNC). Although the cells were not stained in sterile conditions, a lid was kept on the plate wherever possible. During staining the plate was kept on ice, or at 4°C in the centrifuge and all the solutions that were added to the cells were ice cold. This low temperature was maintained to prevent both antibody capping and the staining antibodies from activating the cells. Before staining, the cells were counted with ethidium bromide acridine orange (see section 2.2.1) and diluted to 1 by $10^6$ cells per ml in PBS/BSA (PBS containing 1% bovine serum albumin (BSA) and 0.05% sodium azide (NaN₃)). At least 400ul of this cell suspension was required for staining, because each well received 200ul and each sample was stained in two separate ways, once with the antibody of interest and once with a negative control (therefore two
wells, both containing 200ul were required). A negative control is an antibody of the same subclass and from the same type of animal as the antibody of interest but it is directed against an antigen that does not exist in the test system. Therefore the negative control allows non-specific binding to be quantitated. Once the cells had been added to the plate it was spun at 1500rpm for 5 minutes at 4°C (this allowed slow cooling to 4°C), the supernatant was removed by aspiration and the cell pellet resuspended by agitating the plate. The plate was immediately put on ice and then 20uls of the appropriate antibody was added. The staining antibody, or negative control, was incubated with the cells for 35 minutes to allow binding. The cells were washed three times with PBS/BSA by adding 200ul to each utilised well, spinning the plate as before, removing the supernatant by aspiration and then resuspending the cells. After the final wash, the cells were resuspended in 200uls of 1% paraformaldehyde in PBS. The plate was then wrapped in aluminium foil, and left at 4°C overnight before analysis on a FACScan flowcytometer (Beckton Dickinson) (See section 2.11.4).

This general methodology was used with a variety of different antibodies. The specific cases and how the methodology differed from this general methodology will now be described.

2.6.1 Staining with unconjugated anti-CD3 and anti-CD14

Some of the antibodies used in this study were “unconjugated”, meaning that they were not fluoresceinated and conversely “conjugated” means that they were. To demonstrate that a significant number of cells were being removed during depletion
(see above), the depleted and non-depleted cell samples were stained for CD3 and CD14 straight after the depletion. The possibility exists that cells might bind antibody but not bind a Dynabead. Therefore, a directly conjugated anti-CD3 or CD14 could not be used as some cells might already be bound to the unconjugated antibodies and the latter may inhibit the binding of the former. Four aliquots of the non-depleted sample were used, one pair to stain with anti-CD3 and its negative control and one with CD14 and its negative control. Two aliquots of the depleted samples were used. The T-cell depleted culture was stained with anti-CD3 and its negative control and the monocyte depleted culture was stained with anti-CD14 and its negative control. The cells were stained with anti-CD3 and CD14 at 1ug per million cells as described above. However, as these antibodies are not directly conjugated, a secondary conjugated antibody also needed to be used. Once the excess primary antibody had been washed off, 20ul of a conjugated rabbit anti-mouse immunoglobulin was added. The cells were incubated for 35 minutes on ice and washed three times, as before, with PBS/BSA. After the final wash the cells were fixed in paraformaldehyde as described above. This methodology was also used in the preliminary experiments to assess what concentration of the anti-CD3 and CD14 supersaturated the cells, 10ug, 1ug and 0.1ug per million cells were tested.

2.6.2 Staining with a directly conjugated anti-CD19

In eight of the seventeen patients included in the depletion experiments, the number of B-cells were quantitated in both depleted and non-depleted cultures. This
measurement was performed by staining the cells with a directly conjugated anti-CD19 (as above) and then, using FACs analysis, the percentage of viable PBMC that were B-cells was calculated. The total number of viable cells were then counted using ethidium bromide acridine orange (see above). Using the following formula, the total number of B-cells in each culture were calculated.

\[
\text{% of viable B-cells} \times \text{Number of viable cells} = \text{Number of viable B-cells in the culture}
\]

Invariably, the depleted cultures had fewer B-cells (approximately 20-30% less) than would be expected, due to non-specific loss during depletion. The depleted cultures were spun down at 1500rpm for 10 minutes and resuspended in a volume of supplemented RPMI such that they contained the same concentration of B-cells as the non-depleted cultures.

2.6.3 Double staining with annexin and anti-CD3 to quantitate T-cell apoptosis

An Annexin V-FITC kit (R&D) was used to quantitate the level of apoptosis and a directly conjugated anti-CD3 antibody from Pharmingen was used to label the T-cells. Annexin requires room temperature and calcium ions to bind effectively, so the “general methodology” had to be altered. First the anti-CD3 antibody was used as described in the “general methodology”. Instead of using PBS/BSA to wash off the excess anti-CD3 antibody, annexin buffer (which is rich in calcium and was provided in the Annexin kit) was used on the final two (second and third) washes. The cells
were then resuspended in 100μl of the Annexin buffer, and left on ice for 7 minutes. The cells were then spun at 1500rpm for 5 minutes and the supernatant removed by aspiration. Twenty μl of the annexin buffer and 5μls of the annexin V-FITC were then added to the cells, in that order. The plate was immediately wrapped in aluminium foil and incubated in the dark and at room temperature for 15 minutes before being analysed on the FACscan. The annexin stained cells were not stored, as annexin binding is temporary.

2.6.4 Flow cytometric analysis of mononuclear cells

The stained cell preparations were analysed on a Beckton Dickenson FACScan flowcytometer using Win FCM software. Essentially the mononuclear cells were gated on by forward scatter (FSC) and right angle light scatter (SSC) to discount any debris or polynuclear cells. The cells were analysed for fluorescence staining on FL1 (FITC), FL2 (PE) and FL3 (Cytochrome). A minimum of 20,000 events were acquired for analysis.
Results

3.0 Optimisation experiments

In order to define the best and most appropriate experimental conditions, the following preliminary experiments were undertaken. Primarily an ELISA that could detect the spontaneous anti-dsDNA antibody production of lupus PBMC was required. Subsequently the culture time and cytokine concentration required to show a result that might be biologically relevant was investigated.

3.1 Choice of ELISA methodology

In order to develop an ELISA sensitive enough to detect the anti-dsDNA antibody production from PBMC cultures; a number of protocols were assessed. Each protocol was used to assess the antibody production from the cultures of three patients’ PBMC and the test that returned the highest specific OD was selected.

“Protocol 1”, which was selected, is detailed in section 2.4. Where the other protocols differed from protocol 1, will now be described.

Protocol 2

Double stranded DNA does not bind strongly to ELISA plates. In order to improve this binding, a molecule that binds both ELISA plates and dsDNA was first added to all the wells. 50ul of Poly-L-Lysine (50ug/ml in distilled water) was added to all the wells in a plate and incubated for 1 hour at 37°C. The plate was then washed twice with PBS. The test half of the plate was then coated with 50ul of dsDNA (20ug/ml in
distilled water) and the uncoated side in distilled water. The plate was then incubated for 1 hour at 37°C before being washed three times in PBS. 100ul of poly-L-Glutamate (100ug/ml in distilled water) was added to all wells to neutralise the effect of the highly charged poly-L-Lysine. This was designed to reduce non-specific binding of immunoglobulin to poly-L-lysine. After 1 hour at 37°C the plates were washed three times with PBS and 200ul of 2% casein in PBS was added to each well. This was to decrease non-specific binding of immunoglobulin to plastic. After 1 hour at 37°C the plates were washed 3 times in PBS Tween and the supernatants to be tested were added. From this point on protocols 1 and 2 are identical.

Protocol 3
50ul of dsDNA at 100ug/ml in distilled water was left overnight and uncovered at 37°C on the coated side of the ELISA plate. The plate is then washed three times in PBS. The plate is then blocked with casein as in protocol 2. The ELISA is then completed identically to protocol 1 and 2.

As can be seen from fig 3.1, protocol 1 was marginally more sensitive across the three patients tested (However, statistically there was no difference). Protocol 1 was used as it was technically more straightforward. The antibody levels are expressed as optical densities since expressing them as a percentage of the positive control would confuse the issue of differential sensitivity.
Figure 3.1: Sensitivity of ELISA protocols

**Figure 3.1** PBMC from each patient were separated into three cultures of $2 \times 10^6$ cells per ml in 1ml samples and incubated for three days. Each culture was tested in duplicate with each ELISA protocol described above. The vertical bars represent standard errors. Mann-Whitney revealed no statistically significant differences between the three protocols.

3.2 Example of a dilution curve in an anti-dsDNA ELISA (protocol 1)

As stated in section 2.5, ODs must be in the linear section of the sensitivity range of an ELISA to quantitatively compare antibody levels. Therefore, a serial dilution of a positive control was included on each plate to delineate the linear section on each plate. An example is included, graphing OD against dilution of the positive control (fig 3.2).
Figure 3.2. Dilution of a positive control in an anti-dsDNA ELISA

Figure 3.2. An anti-dsDNA ELISA was undertaken, as described above in protocol 1. A single set of dilutions of the serum from a patient known to be positive for anti-dsDNA antibodies is shown. The figures on the x-axis represent the ratio of dilution (ranging from 1, which was undiluted, to 10000000 [1:1000000]). This curve was regarded as linear between the ODs of 1.0 and 0.18.

3.3 Choice of culture period

Initial time course experiments suggested that there are at least two phases to antibody production in vitro. As can be seen from figure 3.3, antibody level increases most rapidly for the first three to five days and then more slowly for the next ten days. In order to test the effect on only one phase while still achieving measurable levels of anti-dsDNA antibodies, supernatants were taken on day three. Total IgG production
appeared to follow a similar pattern to anti-dsDNA but the initial increase was less rapid and it displayed a later saturation point.

Fig 3.3: Longitudinal study of antibody production

Figure 3.3. PBMC from two patients (BILAG scores 4 and 9) were separated into fourteen cultures of $2 \times 10^6$ cells per ml in $1/2$ ml samples. After days 1, 2, 3, 4, 5, 10 and 15 the supernatants of a pair of cultures were harvested and frozen at $-20^\circ$C. Once all the supernatants had been collected and frozen, each was thawed and tested, in duplicate, for anti-dsDNA and total IgG level by ELISA. Data is expressed as a percentage of the OD achieved at day 15 to allow for the different magnitude of IgG anti-dsDNA and total IgG antibody production. The vertical bars represent standard errors, which were put through the same mathematical transformations.

3.4 Determination of appropriate cytokine concentrations

Many issues complicate selecting the cytokine concentration to use in this study.

Ideally a physiological concentration would be used. However, a physiological concentration is difficult to quantitate as, in vivo, cytokines are targeted over a small area, while existing studies quantitate the cytokine level in serum and supernatant (which are likely to be lower than that surrounding the target cell). In addition, one of the drawbacks of an in vitro model is that the concentration of cytokine used, will to some extent be defined by what concentration has an effect.
Figure 3.4: Titration of cytokine treatment on anti-dsDNA antibody production

PBMC from two patients (BILAG score 3 and 7) were separated into eighteen cultures of $2 \times 10^6$ cells per ml in $1/2$ ml samples. Each cytokine concentration was tested on a pair of cultures and one pair was left untreated. After three days, each supernatant was harvested and tested in duplicate for anti-dsDNA by ELISA. The formula in section 2.5 was used to calculate percentage change in OD and the standard errors (indicated by the vertical bars) were put through the same transformations; this allowed the data from two patients to be graphed together. Mann Whitney revealed that all doses of both IL-10 and IL-12 produced a statistically significant change in antibody production when compared to untreated cultures ($p < 0.05$). There was also a statistically significant difference between the 20ng/ml and 2ng/ml dose of IL-10 ($p < 0.05$) but no difference between 20ng/ml and 100 or 200ng/ml of IL-10. Similarly, there was a statistically significant difference between the 2 and 0.2ng/ml of IL-12 ($p < 0.05$) but no difference between the 10 or 20ng/ml and the 2ng/ml dose.

A range of concentrations around the published EC50 of each cytokine was tested on two patients (Fig 3.4). *In vitro* IL-10 and IL-12 production has been found to be approximately 2ng/ml and 0.2ng/ml in PBMC cultures (Llorente et al. 1993, Horwitz et al. 1998). Therefore 2ng/ml of IL-10 and 0.2 ng/ml of IL-12 were used in these optimisation experiments. The existing literature had used IL-10 at 20 ng/ml (Llorente et al. 1995) and IL-12 at 2ng/ml (Houssiau et al. 1997) therefore these concentrations were also tested. As can be seen from fig 3.4, all the concentrations tested had a large and statistically significant effect on anti-dsDNA antibody
production. In addition there was a statistically significant difference between the lower two concentrations of each cytokine. The effect begins to saturate beyond 20ng/ml of IL-10 and 2ng/ml of IL-12 as indicated by the lack of significant difference between the antibody production of the highest three concentrations tested.

From this study it was decided that 2 and 20ng/ml of IL-10 and 0.2 and 2ng/ml of IL-12 would be used.

The optimisation experiments are critically analysed and discussed in section 4.4.6.
Chapter 4

The effect of interleukin-10 and of interleukin-12 on the in vitro production of anti-double stranded DNA antibodies from patients with systemic lupus erythematosus

Hypothesis

In this chapter the following hypotheses will be tested. 1) IL-10 will increase IgG anti-dsDNA antibody production and 2) IL-12 will decrease IgG anti-dsDNA antibody production.

4.1 Introduction

As described in the Introduction (Chapter 1) there is a great deal of evidence to suggest that anti-dsDNA antibodies are one of the pathogenic factors in SLE and that IL-10 and IL-12 might be important in controlling the levels of anti-dsDNA antibodies. The aim of this first set of experiments was to measure the effect of IL-10 and of IL-12 on the IgG anti-dsDNA antibodies from patients with SLE, before dissecting how they have this effect.

Total PBMC were cultured with IL-10, IL-12 or without cytokine and the supernatants tested for the production of IgG anti-dsDNA and total IgG by ELISA (see methods section 2.1 - 2.4).

It was found that the change in IgG anti-dsDNA antibody production caused by IL-10 correlated weakly with disease activity as measured by the BILAG system. Patients with inactive disease tended to show an increase in IgG anti-dsDNA antibody production while patients with active disease decreased their IgG anti-dsDNA antibody production. The effect of IL-12 on IgG anti-dsDNA antibody production
was less predictable, affecting some patients PBMC differently to others. This effect was termed “inter-patient variation”.

4.2 Patients and controls

4.2.1 Patients

Patient details are summarised in the following text, (see Table 4.1 for more details).

Thirty-one female SLE patients were studied and each met four or more of the revised classification criteria for SLE (Tan et al. 1982). Patients were selected at random from amongst those known to have currently raised serum anti-dsDNA antibody levels and this was retested at the time of visit to the clinic. Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) index.

All patients were tested with IL-10: three Afro-Caribbeans, two Asians, 24 Caucasians and two Chinese; the mean age of this group was 38 years (range 17-70). 21 of these patients had active disease (global BILAG score of > 5, see Introduction) at the time of testing. Twenty-five female patients with SLE were tested with IL-12 (patients 1 - 25 in Table 4.1): three Afro-Caribbeans, two Asians, nineteen Caucasians and two Chinese; the mean age was 38 years (range 17-70). Eighteen of these patients had active disease at the time of testing.

Table 4.1 Patients details

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Years)</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>BILAG score</th>
<th>Drug treatments</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

21 of these patients had active disease. Patients 1 - 25 in Table 4.1: three Afro-Caribbeans, two Asians, nineteen Caucasians and two Chinese; the mean age was 38 years (range 17-70). Eighteen of these patients had active disease at the time of testing.
### Table 4.1 continued

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<td>F</td>
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<tr>
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<td>-</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key and legend for Table 4.1**

- **F** - Female
- **Cyclo** - Cyclophosphamide
- **mg** - milligrammes
- **MTX** - Methotrexate
- **AZA** - Azathioprine
- **Hydrox** - Hydroxychloroquine
- **C’casian** - Caucasian
- **A/C** - Afro-Caribbean
- **mnth** - month
- **Fld** - IL-10 failed to cause a significant (p <0.05) difference in OD (using T-test to compare mean OD from the triplicate untreated cultures versus triplicate treated cultures). **X** - indicates the same but for IL-12
- * By patient number signifies that the patient produced a detectable level of anti-dsDNA antibodies

Table 4.1 The first column numbers patients for the purposes of identification. All patients were tested with IL-10 and patients 1 – 25 were tested with IL-12.
4.2.3 Healthy controls

Sixteen healthy female controls (HC) were used: seven Asians and nine Caucasians; mean age 26 years (range 23-45). All control blood samples were tested with IL-10 and with IL-12.

4.3 Tests completed

All patients were tested for total IgG and anti-dsDNA antibodies. However, many patients did not produce sufficient levels of anti-dsDNA antibodies to be detectable by ELISA. For this reason there is a greater n number presented for total IgG than anti-dsDNA antibody production.

PBMC were separated as described in section 2.1.1. They were cultured at 2 x 10^6 cells per ml in 1/2 ml cultures. Each treatment was tested with triplicate cell cultures and ELISA tested each culture in duplicate. Chapter 3 explained how these experimental conditions were decided upon.

4.4 Results

4.4.1 Spontaneous IgG and specific anti dsDNA production

4.4.1a Total IgG antibody production

All SLE and HC lymphocytes produced measurable quantities of total IgG after 3 days of culture; these are summarised in table 4.2. The differences in spontaneous IgG production between HC and SLE patients with active or inactive disease were highly significant (p<0.001 in both cases, tested by Mann Whitney). However, there
was no significant difference in spontaneous IgG production between SLE patients with active versus inactive SLE.

Table 4.2 Spontaneous IgG production from in vitro cultures

<table>
<thead>
<tr>
<th>Healthy Controls</th>
<th>SLE (inactive disease)</th>
<th>SLE (active disease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 +/- 5.0ng/ml</td>
<td>138 +/- 185ng/ml</td>
<td>402 +/- 298ng/ml</td>
</tr>
<tr>
<td>n = 16</td>
<td>n = 5</td>
<td>n = 26</td>
</tr>
</tbody>
</table>

Table 4.2. Mann Whitney revealed statistically significant differences between the IgG production of both groups of SLE patients and healthy controls (p < 0.001) but no difference between the two groups of SLE patients.

4.4.1b Anti-DNA antibody production

Lymphocytes from none of the HC and only 17/31 (55%) of SLE patients produced measurable levels of anti-dsDNA. Although the anti-dsDNA antibody production from the untreated cultures did not correlate with BILAG global score it did correlate with serum anti-dsDNA antibody level (n = 20, r = 0.7; p = 0.03). In addition no correlation between serum anti-dsDNA antibody level and disease activity (BILAG score) was seen (n = 31).

The production of detectable levels of anti-dsDNA in vitro was not predicted by any of the parameters tested. (Parameters tested were age, ethnicity, drug treatment, BILAG score, individual BILAG system scores, serum C3 level, serum anti-dsDNA level and untreated anti-dsDNA level).
4.4.3 Effect of IL-10 and IL-12 on total IgG produced by lymphocytes from SLE patients

Regression analysis demonstrated that the only parameter that predicted cytokine induced IgG production was untreated antibody production (r values stated below). Other parameters tested were: BILAG score, individual BILAG components, drug treatment, C3 levels and serum anti-dsDNA level.

It may be that the variation in antibody production between patients obscures any effect that IL-10 has on antibody production. In order to focus the analysis on the cytokine induced change in IgG, untreated and cytokine treated antibody level were combined. Change in antibody production was calculated using the formula below (see section 2.5). Prior to using this formula the correlation (Spearman Rank) between untreated and cytokine treated antibody production was assessed. The r value was almost 1 (0.96, 0.93, 0.92, 0.94 for IL-10 [20 and 2ng/ml] and IL-12 [2 and 0.2ng/ml]; respectively), therefore the following formula could be used (see section 2.5).

\[
\text{Mean percentage change in OD} = \frac{(\text{Mean treated OD} - \text{Mean untreated OD}) \times 100}{\text{Mean untreated OD}}
\]

Despite focusing the analysis on the change induced by cytokine, no correlations between any of the parameters tested and change in total IgG production was seen using sequential linear regression analysis. (31 and 25 patients were tested with IL-10)
at 20ng/ml and IL-12 at 2ng/ml respectively). 20 patients were tested with the lower concentrations of each cytokine. In addition, Mann-Whitney showed no significant differences in response to IL-10 or IL-12 in the total IgG produced by HC or SLE lymphocytes using the above formula to correct for the differences in magnitude of antibody production.

**4.4.4 Effect of IL-10 and IL-12 on anti dsDNA produced by lymphocytes from SLE patients**

As will be discussed in the following sections, some parameters were found to correlate with the effect of IL-10 (20 ng/ml) on anti-dsDNA antibody production. Therefore this raw data will be presented, see figure 4.1 and table 4.1 and 4.3.
Figure 4.1a. PBMC were aliquoted into 1/2 ml cultures at 2 X 10^6 cells per ml and incubated for three days. Cultures were split into sets of three; one set left untreated (“Untreated”) and one set treated with 20ng/ml of IL-10 (“Treated”). The supernatants were tested for anti-dsDNA level by ELISA. The numbers shown represent the mean OD levels obtained (all cultures from a single patient were tested on the same ELISA plate). The standard errors are represented by the vertical bars. The x-axis indicates the BILAG score at the time of testing, the dose of cyclophosphamide (Cyclo) in mg/month, methotrexate (MTX) in mg/week, azathioprine (AZA) in mg/day, prednisolone (Pred) in mg/day, hydroxychloroquine (Hydrox) in mg/day. Results of statistical analysis will be presented later. Remaining patients are presented overpage in graph 2.
Figure 4.1b See legend for fig 4.1a
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Ethnicity</th>
<th>Age (Years)</th>
<th>BILAG components</th>
<th>DNA titre</th>
<th>C3 level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C’casian</td>
<td>46</td>
<td>C C E D D D D D</td>
<td>378.00</td>
<td>1.16</td>
</tr>
<tr>
<td>2</td>
<td>A/C</td>
<td>32</td>
<td>C C E E E E D C</td>
<td>94.00</td>
<td>.52</td>
</tr>
<tr>
<td>9</td>
<td>C’casian</td>
<td>43</td>
<td>C B D D D C E D</td>
<td>130.00</td>
<td>1.27</td>
</tr>
<tr>
<td>10</td>
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<td>.95</td>
</tr>
<tr>
<td>11</td>
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<td>28</td>
<td>B B D D E D D E</td>
<td>562</td>
<td>1.10</td>
</tr>
<tr>
<td>14</td>
<td>C’casian</td>
<td>43</td>
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<td>230.00</td>
<td>.85</td>
</tr>
<tr>
<td>16</td>
<td>C’casian</td>
<td>36</td>
<td>C C C C D C E C</td>
<td>270.00</td>
<td>1.10</td>
</tr>
<tr>
<td>17</td>
<td>Chinese</td>
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<td>.81</td>
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<td>1.31</td>
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<tr>
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<td>.80</td>
</tr>
<tr>
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<td>1.56</td>
</tr>
<tr>
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Table 4.3

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<th>Patient ID</th>
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<th>Age (Years)</th>
<th>BILAG components</th>
<th>DNA titre</th>
<th>C3 level</th>
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<tr>
<td>1</td>
<td>C’casian</td>
<td>46</td>
<td>C C E D D D D D</td>
<td>378.00</td>
<td>1.16</td>
</tr>
<tr>
<td>2</td>
<td>A/C</td>
<td>32</td>
<td>C C E E E E D C</td>
<td>94.00</td>
<td>.52</td>
</tr>
<tr>
<td>9</td>
<td>C’casian</td>
<td>43</td>
<td>C B D D D C E D</td>
<td>130.00</td>
<td>1.27</td>
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<tr>
<td>10</td>
<td>C’casian</td>
<td>38</td>
<td>C D C C D D D D</td>
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<tr>
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<tr>
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<td>16</td>
<td>C’casian</td>
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<td>C C C C D C E C</td>
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<td>1.10</td>
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<tr>
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<td>Chinese</td>
<td>22</td>
<td>C C C D B C E C</td>
<td>298.00</td>
<td>.81</td>
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<tr>
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<td>B C D B D C E C</td>
<td>250.00</td>
<td>.93</td>
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<tr>
<td>19</td>
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<td>B B C C E C E D</td>
<td>117.00</td>
<td>1.42</td>
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<tr>
<td>20</td>
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<td>B D C D B C C B</td>
<td>650.00</td>
<td>.70</td>
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<tr>
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<td>.85</td>
</tr>
<tr>
<td>28</td>
<td>A/C</td>
<td>26</td>
<td>B B D B C B E D</td>
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<td>1.25</td>
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<td>.80</td>
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<tr>
<td>30</td>
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<td>23</td>
<td>B D D C B E A C</td>
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<td>1.56</td>
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<td>31</td>
<td>C’casian</td>
<td>25</td>
<td>A D D D C B D C</td>
<td>90.00</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Table 4.3

- C’casian – Caucasian
- A/C – Afro-Caribbean
- CNS – central nervous system
- Musc – musculoskeletal
- CVS – cardiovascular and respiratory
- DNA – serum anti-dsDNA titre
  - (Considered normal < 50u/ml) C = 1
- C3 – complement 3 level
  - (Considered normal: 0.98 – 1.8g/l) D and E = 0
- Haem – haematological
  - A = 9
- DNA titre
- C3 level
Due to the large number of parameters shown it is difficult to visualise any relationships. Therefore, a sequential regression analysis was used to test which, if any parameters predicted anti-dsDNA antibody OD from IL-10 treated cultures. (Parameters tested were age, ethnicity, drug treatment, BILAG score, individual BILAG system scores, serum C3 level, serum anti-dsDNA level and untreated anti-dsDNA level). Untreated antibody level was strongly predictive of treated antibody level \((r = 0.9, \ p = 0.02, \ n = 17)\). With treated antibody level as the dependent variable, both BILAG score and hydroxychloroquine treatment were weakly predictive of IL-10 (20ng/ml) treated antibody level (see table 4.4 for statistical analysis). However, only hydroxychloroquine treatment was predictive when both were included, suggesting that it had the stronger effect. The beta values and p values of each drug treatment and BILAG score are shown in table 4.4. All the patients tested were on multiple treatments, therefore when it is stated that a patient was on a drug (for example hydroxychloroquine) the patient may also be on other drugs (for example methotrexate). Only untreated antibody level predicted the antibody production from IL-10 (2ng/ml) treated cultures \((r = 0.09, \ p = 0.03, \ n = 11)\).

### Table 4.4 Relationship between IL-10 treated OD and the parameters examined

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(\beta) value</th>
<th>(p) value</th>
</tr>
</thead>
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<tr>
<td>Untreated OD</td>
<td>0.9</td>
<td>0.024</td>
</tr>
<tr>
<td>BILAG</td>
<td>-0.559</td>
<td>0.032</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>0.606</td>
<td>0.023</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.606</td>
<td>0.235</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>0.336</td>
<td>0.118</td>
</tr>
<tr>
<td>Prednisolone (\geq)10mg/day</td>
<td>0.435</td>
<td>0.109</td>
</tr>
<tr>
<td>Prednisolone &lt;10mg/day</td>
<td>0.302</td>
<td>0.218</td>
</tr>
</tbody>
</table>

### Table 4.4 Relationship of each drug treatment and BILAG score with the anti-dsDNA antibody level in IL-10 (20ng/ml) treated cultures.
4.4.4b Demonstrating the relationship between IL-10 induced change in antibody production and BILAG score

To best show this relationship in a graph, difference in OD was plotted against BILAG score (mean IL-10 treated OD – mean untreated OD). For comparison of raw data see fig 4.1. Mean difference in anti-dsDNA antibody production correlated with disease activity, $r = -0.48$, $p = 0.042$ (Fig 4.2); such that patients with low disease activity scores had increased antibody production whilst patients with high disease activity scores had decreased antibody production ($n = 17$).

4.4.4c Demonstrating the relationship between IL-10 induced antibody production and hydroxychloroquine treatment

As stated above, hydroxychloroquine treatment predicted IL-10 (20ng/ml) treated antibody production, see table 4.3. In order to demonstrate this graphically, patients were subdivided by which treatments they were taking (regardless which other treatments they were also taking). Comparing change in antibody production (expressed as treated OD – untreated OD) of those patients on a drug treatment (for example hydroxychloroquine) with those not on that drug treatment (any treatment regime that did not include, in this example, hydroxychloroquine) revealed no obvious differences (see fig 4.3). This was due to the large standard errors caused by the co-existence of positive and negative differences in the same group.

For further analysis patients were subdivided by which treatments they were taking (regardless which other treatments they were taking). Within each group the number of patients that increased their antibody production were compared to the
number that decreased their antibody production. This allowed the differences by
treatment to be more easily visualised (see fig 4.4). The observation that nine
increased and only one patient decreased their antibody production when on
hydroxychloroquine, while the other drug groups had a more equal distribution of
“increasers” and “decreasers” may demonstrate why hydroxychloroquine treatment
was predictive. Using the assumption that the expected distribution of increasers
versus decreasers would be equal, chi squared test revealed a statistically significant
difference from the expected in only the hydroxychloroquine treated group (p = 0.02).
This observation will be further explored in the discussion.

4.4.4d IL-12
Sequential regression analysis of each parameter described in table 4.1, 4.2 and figure
4.1 failed to reveal any statistically significant associations with IL-12 induced
change in anti-dsDNA. (Parameters tested were age, ethnicity, drug treatment,
BILAG score, individual BILAG system scores, serum C3 level and serum anti-
dsDNA level). Focussing the analysis by using percentage change in OD (see section
2.5 and section 4.4.3), also failed to demonstrate any significant relationships.
Figure 4.2. Cultures were set-up at $2 \times 10^6$ cells per ml in $0.5$ ml cultures, supernatants were taken on day 3. Each point is the mean difference between three pairs of cultures, one of each pair being treated with 20ng/ml of IL-10 and one left untreated. All cultures were tested in duplicate and each pair was tested on the same ELISA plate. Spearman rank revealed a statistically significant correlation, $r = -0.48$, $p = 0.042$, $n = 17$. 

**Fig 4.2** Relationship between disease activity and IL-10 induced change in anti-dsDNA.
Figure 4.3. Cyclo – cyclophosphamide, MTX – methotrexate, AZA – azathioprine, Hydrox – hydroxychloroquine.

Legend of fig 4.1 describes the experimental set-up. Patients were counted as taking a drug regardless of dose and what other drugs the patient was taking. Mean difference was equal to mean IL-10 treated OD minus mean untreated OD, vertical bars represent the standard error. Mann Whitney revealed no statistically significant differences between the groups.
Figure 4.4 Anti-dsDNA antibody response of patients when grouped by drug treatment.

Figure 4.4 Cyclo - cyclophosphamide, MTX - methotrexate, AZA - azathioprine, Hydro - hydroxychloroquine. Each pair of columns contains all patients in the cohort who were taking the labelled drug, regardless at what dose and which other drugs were in their regime. Chi squared test was used to compare the distribution of "increasers" to "decreasers" in each treatment group. The only one statistically significant difference was in the hydroxychloroquine group (p = 0.02).
4.4.5 The effects of cytokines on IgG anti-dsDNA antibody production, repeated testing on five patients

Disease activity and hydroxychloroquine treatment appeared to be weakly linked to the responsiveness of lupus PBMC to IL-10 at the 20ng/ml concentration. None of the parameters tested appeared to correlate with the effect of IL-12 at the concentrations used. In an attempt to control for inter-patient variability (and to test the effect of inter-patient variation) it was decided to test a small group of patients repeatedly. Five patients who were regularly attending the lupus clinic with active lupus and raised serum anti-dsDNA antibody levels were selected. Each was tested on five occasions over the course of one year with the higher concentrations of cytokine used (20ng/ml of IL-10 and 2ng/ml of IL-12). Table 4.5 gives the patient details and figure 4.5 shows the raw data obtained.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age (years)</th>
<th>Ethnicity</th>
<th>BILAG score</th>
<th>Drug treatments</th>
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<td></td>
<td></td>
<td></td>
<td>Cyclo mg/mnth</td>
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<tr>
<td>A1</td>
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<td>2</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>A5</td>
<td>8</td>
<td>-</td>
<td>10</td>
<td>-</td>
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<tr>
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### Table 4.5 continued

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<th>Patient ID</th>
<th>Age (years)</th>
<th>Ethnicity</th>
<th>BILAG score</th>
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<th>AZA mg/day</th>
<th>Pred mg/day</th>
<th>Hydrox mg/day</th>
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<td>-</td>
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<td>10</td>
<td>-</td>
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<td>11</td>
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<td>150</td>
<td>7.5</td>
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<td>12</td>
<td>-</td>
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<td>125</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>12</td>
<td>750</td>
<td>10</td>
<td>150</td>
<td>20</td>
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<td>-</td>
<td>100</td>
<td>5</td>
<td>200</td>
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<td>6</td>
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<td>E3</td>
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<td>125</td>
<td>7.5</td>
<td>400</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>125</td>
<td>7.5</td>
<td>400</td>
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<td>-</td>
<td>-</td>
<td>150</td>
<td>10</td>
<td>400</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Key and legend for Table 4.5**

A/C - Afro-Caribbean
C’casian - Caucasian
mnth - month
Cyclo - Cyclophosphamide

The first column identifies the patients. The letter corresponds to the patient, the number to the visit; therefore E1 indicates patient E, on their first visit.

#### 4.4.5a IL-10 (20ng/ml)

Sequential regression analysis of each parameter revealed some statistically significant associations, see table 4.6. (The parameters tested were age, ethnicity, drug treatment, BILAG score, individual BILAG system scores, serum C3 level, serum anti-dsDNA level and "inter-patient variation" [described in introduction] which was analysed by entering patient ID number into the regression analysis).

In this cohort of patients BILAG score remained associated with IL-10 treated antibody level; however hydroxychloroquine treatment did not. Interestingly untreated antibody production was no longer predictive of treated antibody production and neither was inter-patient variation.
Fig 4.5a IL-10 longitudinal data, graph 1

Fig 4.5a See legend for fig 4.5b
Figure 4.5b. cyclophosphamide (cyclo) in mg/week, methotrexate (MTX) in mg/day, Azathioprine (AZA) in mg/day, prednisolone (pred) in mg/day, hydroxychloroquine (hydrox) in mg/day. See legend of figure 4.2 for details of experimental set-up. Statistical analysis is shown later.
Figure 4.5c. See legend of figure 4.5d
Figure 4.5d. Cyclophosphamide (cyclo) in mg/week, methotrexate (MTX) in mg/day, Azathioprine (AZA) in mg/day, prednisolone (pred) in mg/day, hydroxychloroquine (hydrox) in mg/day. See legend of figure 4.2 for details of experimental set-up. IL-12 was used at 2ng/ml and administered to the cultures identically as described with IL-10. Statistical analysis is shown later.
4.4.5b Inter-patient variation

Inter-patient variation was tested by giving each patient a number (1-5) and including this number in the regression analysis. The failure of this parameter to correlate with IL-10 treated OD suggests that inter-patient variation does not predict the effect of IL-10 on anti-dsDNA antibody production.

Table 4.6. Relationship between IL-10 treated OD and the parameters examined

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β- value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BILAG</td>
<td>-0.464</td>
<td>0.02</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>-0.03</td>
<td>0.998</td>
</tr>
<tr>
<td>Untreated antibody level</td>
<td>0.3</td>
<td>0.174</td>
</tr>
<tr>
<td>Inter-patient variation</td>
<td>-0.036</td>
<td>0.852</td>
</tr>
</tbody>
</table>

Table 4.6. β value is the standardised co-efficient and p the probability of the relationship being due to chance as calculated by a regression formula.

4.4.5c Demonstrating the relationship between IL-10 (20ng/ml) and BILAG score

To demonstrate optimally how the effect of IL-10 on anti-dsDNA antibody production varied with disease activity, that effect was calculated as difference in antibody production (Untreated OD – IL-10 treated OD) and graphed against BILAG score see figure 4.6, b and c.

With increasing disease activity there was a trend towards a decrease in IgG anti-dsDNA antibody production (fig 4.6a, b and c). In 3/5 of the patients there was an increase in IgG anti-dsDNA antibody production at lower disease activities; this was consistent with the data obtained in the cross-sectional study.

4.4.5d IL-12 (2ng/ml)

Sequential regression analysis of each parameter revealed that the only parameters that predicted IL-12 treated anti-dsDNA antibody production were inter-patient
variation and untreated OD, see table 4.7. (The parameters tested were age, ethnicity, drug treatment, BILAG score, individual BILAG system scores, serum C3 level, serum anti-dsDNA level and inter-patient variation).

Table 4.7. Relationship between IL-12 treated OD and the parameters examined

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β- value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated OD</td>
<td>0.616</td>
<td>0.002</td>
</tr>
<tr>
<td>Inter-patient variation</td>
<td>0.399</td>
<td>0.038</td>
</tr>
<tr>
<td>BILAG</td>
<td>-0.356</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 4.7. β Value is the standardised co-efficient and p the probability of the relationship being due to chance as calculated by a regression formula.

4.4.5e Longitudinal relationship between IL-12 (2ng/ml) and anti-dsDNA antibody production

As inter-patient variation predicted treated antibody production it appeared that IL-12 was having either no effect, or a subtle effect. In an attempt to pick out any effect of IL-12, percentage change in anti-dsDNA antibody production was graphed against BILAG score (See section 2.5). This would focus the analysis on the effect of IL-12 and correct for the variation in antibody production between visits (See fig 4.6d - f).

In four of the five patients tested at the different time points, there was a tendency towards a decrease in IgG anti-dsDNA antibody production with increased disease activity (Fig 4.6d - f). However, there was one patient, E, who showed an enhancement of IgG anti dsDNA antibody production at the higher disease activities reached in this patient. When change in antibody production was correlated with global score this data approached but did not reach significance ($r = -0.356$, $p = 0.08$).
Fig 4.6a Difference in antibody response to IL-10, graph 1

Fig 4.6a See legend for figure 4.6c
Fig 4.6b. See legend for fig 4.6c
Fig 4.6c Difference in antibody response to IL-10, graph 3

See figure 4.2 legend for details of experimental set-up and analysis. Spearman rank analysis revealed a significant relationship between difference in OD and disease activity ($r = -0.464$, $p = 0.02$).
Fig 4.6d. See legend for fig 4.6f.
Fig 4.6e Difference in antibody response to IL-12, graph 2

Fig 4.6e. See legend for fig 4.6f
Fig 4.6f See figure 4.2 legend for details of experimental set-up and analysis. IL-12 was tested at 2ng/ml. Spearman rank analysis revealed no significant relationship between % change in antibiotic production (see section 2.6) and disease activity ($r = -0.356$, $p = 0.08$).
4.5 Discussion

4.5.1 Summary of results

In this chapter it has been confirmed that in vitro spontaneous IgG production of PBMC from patients with SLE is significantly higher than that produced by PBMC from HC. However, neither IL-10 (at 2 and 20ng/ml) nor IL-12 (at 0.2 or 2ng/ml) significantly altered this production.

A high proportion of patients with SLE spontaneously made sufficient anti-dsDNA IgG, in vitro, to be able to measure the effect induced by IL-10 and IL-12. The anti-dsDNA response to IL-10 at 20ng/ml correlated with disease activity and with hydroxychloroquine treatment. The correlation with disease activity, but not hydroxychloroquine treatment, was repeated in the five individual patients studied at five different time points. No significant correlations were found with anti-dsDNA antibody production in response to 2ng/ml of IL-10, beside untreated anti-dsDNA antibody production.

Only untreated OD correlated with the effect of IL-12 on anti-dsDNA antibody production in the cross-sectional study. In the five individual patients tested on five separate occasions, only inter-patient variation and untreated anti-dsDNA antibody OD were predictive of IL-12 treated OD, although BILAG score approached significance.
4.5.2 Criticism of the experiments in chapter 4

4.5.2a Patient selection

In order to maximise the chances of testing patients that would produce detectable levels of anti-dsDNA in vitro, patients with known high serum levels of anti-dsDNA were tested. This selection in itself skews the data. In addition only the patients that produced detectable levels of anti-dsDNA were included in the analysis, further skewing the data. As no parameter could be found that predicted which patients would produce detectable levels of anti-dsDNA, it was not possible to pursue this question using this study as a foundation.

4.5.2b Venesection

Blood was taken into heparin containing tubes. As some anti-dsDNA antibodies cross-react with heparin sulphate (Madaio 1999) it may be that its presence could alter the anti-dsDNA response. Other anti-coagulants could have been assessed, for example citrate.

4.5.2c Separation of PBMC

Histopaque was the only method of cell separation employed in this study. As a cytotoxic substance it may have had an effect on the PBMC. If it altered the apoptotic profile of the cells it may have altered the availability of dsDNA and therefore of the anti-dsDNA level. Experiments on whole blood could have been attempted, though such experiments would have had a radically different cytokine milieu.
4.5.2d Culture method

The optimum concentrations of cells and the volume of medium were not examined. Indeed it may have been that the effect of cytokines changed with the concentration of cells. The number of cell-cell interactions is likely to be related to cell concentration and cytokines exert some of their actions at this point.

Experiments on optimum time period were undertaken. However, this was done purely from the standpoint of what length of time optimised antibody level. The optimal time to administer cytokine was not considered. This will be discussed further in section 4.5.4.

The concentration of cytokine added was evaluated on an empirical level to discover what concentration caused a measurable effect. The problems inherent in selecting a concentration were discussed in section 3.4. However, no effort was made to test the dynamic of cytokine concentration, for example divide the dose over time or to test whether the concentration decayed appreciably over time.

4.5.2e Measurement of result

Only ELISA was used to evaluate anti-dsDNA level. The use of other techniques would have rendered the data more convincing. Assessment of anti-dsDNA by a method that only detects high affinity antibodies (Farr assay) would have been useful to make inferences about disease activity and possible pathogenicity of those antibodies signalled to expand.

4.5.2f Analysis of data

In order to express change in antibody production, mean difference and mean percentage change were calculated. When using mean difference, some of the
absolute differences were small. Preliminary experiments should have been undertaken to show the resolution of the ELISA used; as it may be that some of the differences shown were smaller than could be detected by the ELISA system used.

There are limits that any stimulus of change in antibody production will reach; for example the number of B-cells capable of producing the antibody of interest versus the percentage of B-cells spontaneously producing that antibody. Mean percentage difference attempts to allow for those constraints (although it would have been better to systematically investigate those constraints directly). However, it will also conceal the absolute difference in OD. The discussion within this paragraph makes the assumption that cytokines will affect antibody production. In order to nullify this criticism raw data were used in the statistical analysis (See tables 4.4 and 4.6). However, the actual magnitude of the differences found were not verified empirically.

The statistical analysis employed in this chapter is perhaps questionable given the number of possible parameters that were involved. In order to assess which of the possible independent variables might predict cytokine treated antibody production regression analysis was repeated for each variable. (The parameters tested were age, ethnicity, drug treatment, BILAG score, individual BILAG system scores, serum C3 level, serum anti-dsDNA level and inter-patient variation). The number of patients tested does not justify this number of analyses and therefore increases the chance of making a statistical error. To assess each of these parameters properly a study of hundreds of patients would be required. In addition, it must be stated that the statistical associations found were weak, although they did satisfy the $p < 0.05$
threshold that is conventionally used in biological systems. However, some statisticians believe that when employing the more sensitive statistical tests, such as regression, the more discriminating threshold of $p < 0.01$ should be used. With the exception of untreated antibody production, none of the beta values found were convincing (not being close to one). Therefore the tests indicate that there is a moderate chance that there is a weak association.

4.5.3 Relationship between untreated and treated OD, does cytokine treatment effect antibody production?

When five patients were tested repeatedly the mean untreated OD was not predictive of treated OD. This observation suggested that IL-10 was having a significant effect on this cohort of patients. This may be due to chance selection of an IL-10 responsive subgroup of patients. Conversely, it may be that the inclusion of multiple comparisons of untreated versus treated OD within the same patient may allow the effect of IL-10 to become more obvious. Indeed, when this is done within the cross-sectional study, (by comparing the triplicate treated with the untreated cultures within one patient), many patients showed a significant difference (13/17 using paired T-test, those patients that showed a significant difference are indicated on table 4.1).

However, chance pairing of certain treated with untreated cultures may confer a significant difference whereas other combinations of comparison may not.

Sequential regression analysis of each parameter tested failed to find one that predicted which patients might respond to IL-10.

Inter-patient variation and untreated OD was predictive of IL-12 treated antibody production. This result suggested that IL-12 had a smaller effect than the
variation between patients and within a patient at any given time; thus bringing doubt as to whether IL-12 has any measurable effect in these conditions. When the triplicate treated cultures were compared with the triplicate untreated cultures as three paired samples, 8/13 patients showed a significant difference (using paired T-test).

4.5.4 Effect of IL-10 and IL-12 on the antibody production from healthy controls

The majority of other studies that have assessed the impact of cytokines on antibody production have done so in conjunction with other stimuli. They appear to show that B-cells respond to stimulation by either proliferating or maturing into plasma or memory cells; perhaps mirroring their in vivo life cycle.

Human peripheral blood B-cells require initial stimulation to become sensitive to maturational signals, most often CD40 or surface immunoglobulin (sIg) ligation is used. After 3-4 days most cells become CD38+ and, it is thought, cytokine sensitive. The cytokine receptor staining antibodies are, as yet, not suitably characterised to undertake definitive studies, however, empirically B-cells become cytokine responsive at this stage (Arpin et al. 1995, Lui and Banchereau 1997).

Continued stimulation with CD40 or sIg results in increased antibody production at approximately day seven to eight. Combined stimulation through both CD40 and BCR results in delaying the expansion of immunoglobulin to day 14, with increased proliferation taking place in the interim (Burkin et al. 1995, Schilizzi et al. 1997, Banchereau et al. 1994).

The addition of IL-10 to CD40 or sIg stimulated B-cells increased immunoglobulin production of the IgG subclass, particularly when IL-2 was co-administered, but did not alter the time frame of this expansion. Also, addition of an
IL-10 neutralising antibody on day four of a CD40 or sIg stimulated culture diminished the *in vitro* IgG antibody level at day eight (Kindler and Zubler 1997, Fluckiger et al. 1993).

The comparison of these cultures with lupus cell cultures is not straightforward. It seems possible that the failure to pre-stimulate the cells may explain the absence of response to cytokine in total IgG in both SLE patients and healthy controls. The observation that some patients with SLE responded to IL-10 may suggest that those cells were appropriately pre-stimulated *in vivo*.

Other studies have demonstrated that lupus PBMC have a higher than normal level of activated cells (Spronk et al. 1996). CD40 expression is abnormally high in patients with active SLE and low in patients with inactive disease (Datta et al. 1996). The levels of all cytokines are deranged and high in active disease, but are often normal in inactive disease (See tables 1.4, 1.5 and 1.6).

In broad terms, the observation of a variable response to IL-10 is difficult to explain in the light of the published literature; as IL-10 tends to augment antibody production (Kindler and Zubler 1997, Fluckiger et al. 1993). It may be that IL-10 is acting through another mechanism in patients with active disease. Conversely, there is a precedent for multiple stimulation resulting in proliferation in favour of immunoglobulin production (Schilizzi et al. 1997 and Banchereau et al. 1994).

Unfortunately this possibility has not been tested. No studies of the effect of IL-12 on antibody production from human healthy controls could be found.
The use of mixed cell cultures and the omission of baseline measurements of activation markers (CD40, CD38 and cytokine levels, especially IL-2 given the use of IL-10) constitute major criticisms of this study. No effort was made to pre-stimulate cells, to inhibit IL-10 or to add IL-10 at a later stage to ascertain when lupus cells might be equivalently activated as pre-stimulated B-cells from HC. Combined, the failure to complete these standardisation experiments render any meaningful comparisons with the published literature impossible.

4.5.5 Effect of IL-10 in patients with SLE

Several studies have shown that cultures of peripheral blood lymphocytes from SLE patients have increased spontaneous IgG and in particular anti-dsDNA antibody production in vitro by ELISA (Llorente et al. 1995, Spronk et al. 1996) and by ELISPOT (Houssiau et al. 1997), especially in patients with high disease activity. Since increased levels of IL-10 have been found in the serum of patients with SLE which correlate with disease activity using the SLEDAI index (Park et al. 1998) it was important to determine the role of this cytokine in the production of spontaneous antibodies, in vitro. It has been shown that the amount of IL-10 produced spontaneously by SLE monocytes and B cells in vitro is 33 times higher than that produced by control cells (Llorente et al. 1993). Furthermore, the PBMC from relatives and spouses of some patients also produce increased amounts of IL-10, in vitro, (Llorente et al 1997, Grondal et al. 1999). Inclusion of anti IL-10 into cultures has been shown to inhibit spontaneous IgG and anti dsDNA antibodies (al-Janadi et al. 1996) whereas inclusion of IL-10 increases IgG production independently of
disease activity, in 5 day, T-cell depleted cultures (Llorente et al. 1995). It is not clear why total IgG antibody production did not change on addition of IL-10. It is possible that had the cells been cultured for 5 days, as opposed to 3, the changes in IgG may have been more significant. However, 3 day cultures were chosen for the analysis of anti-dsDNA antibodies for reasons explained earlier (see chapter 3).

The effect of exogenous IL-10 on in vitro anti-DNA antibody production has not previously been described. There was a statistically significant correlation between the effect of the higher concentration of IL-10 on anti-dsDNA and disease activity measured on a global scale and with hydroxychloroquine treatment (Figs 4.5 and 4.6). To study this phenomenon better a group of five patients with SLE were tested repeatedly. In this second cohort of patients disease activity remained predictive of IL-10 treated anti-dsDNA antibody production, but hydroxychloroquine treatment did not.

The mechanism by which hydroxychloroquine ameliorates SLE is uncertain. It has been observed that it decreases the level of apoptotic fragments in in vitro cultures of lupus PBMC. This mechanism has been shown to be monocyte dependent. It has been suggested that it may interfere with antigen presentation through monocytes and therefore decrease activation induced cell death. However, the effect of hydroxychloroquine on the activation state of lupus PBMC has not been investigated, nor the effect of fas blockade, nor any other mechanism tested (Liu et al. 2001). If it is the case that hydroxychloroquine decreases apoptosis, it would suggest that increased apoptosis in the IL-10 treated PBMC from patients with active disease
may be responsible for the reduction in anti-dsDNA. In addition, that hydroxychloroquine can block the pro-apoptotic pathway utilised by IL-10. Conversely, it may be that the other drugs (methotrexate, azathioprine, cyclophosphamide) have a more potent effect and can block IL-10 induced antibody production, whereas hydroxychloroquine cannot. No studies documenting the \textit{in vitro} effect of methotrexate, azathioprine or cyclophosphamide on antibody production could be found.

The enhancement effect seen at low disease activity is consistent with the enhancement of total IgG antibody production seen by Llorente \textit{et al} (1995) and the studies in HC. However, the suppressive effects of IL-10 on anti-DNA antibodies in active patients have not been described before. Llorente \textit{et al} (1995) looked at the effect of IL-10 on T-cell depleted cultures and the inclusion of T-cells may have modulated the effects of the IL-10. In fact, the contribution that T cells have to the effects of IL-10 in these experiments is unclear. There is some evidence for a T cell contribution to the production of spontaneous autoantibodies \textit{in vitro}. Early experiments in this field showed that both CD4 and CD8 T cells contributed to this production (Linker-Israeli \textit{et al.} 1990). In addition, T cells expressing neither CD4 nor CD8 i.e. double negative T cells have been shown to “help” the production of spontaneous autoantibodies by SLE PBMC \textit{in vitro} (Datta \textit{et al.} 1997). In more recent experiments CD4+RO+ T cells were shown to produce IL-10 and therefore thought to contribute in this way (al-Janadi \textit{et al.} 1996). However, other experiments have shown that SLE T cells produce little IL-10 (Llorente \textit{et al.} 1993). It is clearly
important to compare the effects of IL-10 with and without added T cells on both total IgG and anti-dsDNA antibody production.

It was not surprising that no effects on anti DNA antibody production were seen at the lower dose of IL-10 (2ng/ml), as this level of IL-10 is spontaneously produced by SLE PBMC in culture (Llorente et al. 1997) and it is thought that change in cytokine concentration causes that cytokine to have an effect (Ding and Shevach 1992).

As stated in the introduction IL-10 has been shown to be capable of inhibiting certain immune responses when T-cells and APCs are present together; perhaps the findings presented in this chapter are another example of this phenomenon.

4.5.6 Effect of IL-12

PBMC from patients with SLE have been shown to produce less spontaneous IL-12, in vitro, than healthy controls (Horwitz et al. 1997, Lui et al. 1998a), this appears to be due to increased IL-10 output which inhibits production of IL-12 by monocytes (Lui et al. 1998b). In this study exogenous IL-12 was added with the aim of reversing the balance of a predominantly Th2 related cytokine, IL-10, to that of a Th1 predominant cytokine, IL-12. Addition of 0.2ng/ml of IL-12 had no overall effect on IgG production or anti-dsDNA antibody production. IL-12 at 2 ng/ml did inhibit anti-dsDNA antibody production in most of the patients in the longitudinal study, particularly at higher disease activities but this correlation did not reach significance (p = 0.08).
Houssiau et al. (1997) found that the addition of 2ng/ml of IL-12 to PBMC cultures from SLE patients decreased the number of anti-DNA antibody producing B-cells as measured by ELISPOT. This result appears to be compatible with the 2ng/ml of IL-12 findings. However, treatment with IL-12 at 2ng/ml increased the anti-dsDNA antibody production of one patient (fig 4.6d).

Preliminary experiments using PBMC from three patients have shown an increase of IL-10 in culture following the addition of IL-12. Up to 167pg/ml of IL-10 has been detected following culture with IL-12 at 2ng/ml but none was detected in response to IL-12 at 0.2ng/ml. Indeed IL-12 has been shown to induce IL-10 production from T-cell clones (Gerosa et al. 1996). This difference in the ability of these two concentrations of IL-12 to induce IL-10 may explain their contrasting effects on antibody production. An effect on antibody production that is reliant on the induction of other cytokines may take some time to develop. This may explain why Houssiau et al. (1997) did not detect this effect in their 16 hour cultures. The mechanism by which IL-12 effects in vitro antibody production requires further analysis.

4.5.7 Conclusion
In conclusion, the relationship between IL-10, IL-12 and antibody production are likely to be complex since autoantibody production is the result of interaction with different cytokines acting on populations of cells at different stages of activation depending on disease activity. Therefore the hypotheses stated in the introduction are too simple to explain the empirical picture.
Chapter 5

The role of T-cells and monocytes in the modulation of antibody production by IL-10.

Hypothesis 1: T-cell depletion will reverse the inhibitory effects of IL-10 on the anti-dsDNA antibody production from the PBMC of patients with SLE who have higher disease activity.

Hypothesis 2: Monocyte depletion will abrogate the effect of IL-10 on the anti-dsDNA antibody production from the PBMC of patients with SLE

5.1 Introduction

Since it was found that IL-10 had a variable effect on IgG anti-dsDNA antibody production from the PBMC of patients with SLE (Chapter 4), further investigation was required to find the factors responsible for this variation.

The effect of IL-10 on antibody production correlated with disease activity, but varied in that patients with inactive disease increased their antibody production while patients with active disease decreased their antibody production. The effect of IL-12 was less predictable, particularly at the higher concentration tested (2ng/ml), where inter-patient variation appeared to interact with its effect on antibody production. As the effect of IL-12 appeared to be more complex and less clear cut than that of IL-10 it was decided to prioritise research into the latter.

Llorente et al (1995) tested the effect of IL-10 on T-cell depleted cultures from SLE patients and found that it increased IgG antibody production regardless of disease activity. In addition it has been shown that T-cells are involved in anti-
dsDNA antibody production (Linker-Israeli et al. 1990). Together these observations raised the possibility that T-cells might be responsible for the disease dependent variation in IL-10 responsiveness demonstrated in chapter 4. To test this possibility, the effect of IL-10 on the antibody production from both total PBMC and T-cell depleted cultures were tested.

The effect of IL-10 in many in vitro systems has been shown to be dependent on the presence of monocytes (De Waal Malefyt et al. 1993). Therefore the effect of IL-10 on antibody production was tested after monocyte depletion.

Total PBMC and PBMC depleted of either T-cells or monocytes were cultured with or without IL-10 and the supernatants tested for their level of anti-dsDNA and total IgG antibodies by ELISA. Pan anti-mouse coated magnetic beads and a murine anti-CD3 and CD14 antibody were used to remove the T-cells and monocytes respectively (see section 2.1.2 Materials and Methods).

T-cell depletion reversed the inhibitory effects of IL-10 at high disease activities without changing its augmenting effect at low disease activities. Monocyte depletion did not significantly effect anti-dsDNA antibody production.

5.2 Patients

Forty two patients with SLE were tested, of whom nineteen produced a sufficient quantity of anti-dsDNA to be measurable by ELISA. Each of the SLE patients studied met four or more of the revised classification criteria for SLE (Tan et al. 1982).

Patients were selected at random from amongst those known to have currently raised serum anti-dsDNA antibody levels. The serum levels were re-assessed at the time of visit to the clinic. Disease activity was assessed using the British Isles Lupus...
Assessment Group (BILAG) index (as described in section 1.1.3, BILAG questionnaire in appendix).

Of the nineteen patients who produced detectable levels of anti-dsDNA, eighteen of these patients were female: three were Asians, three Afro-Caribbeans, eleven Caucasians and one was Chinese. The mean age of this group was 36 years (range 22-64). The remaining male patient was Caucasian and 33 years old. Patient details will be summarised in table 5.1.

Three of the patients (one with active disease, BILAG score of 13) were on high dose steroids (>10mg prednisolone), two (with inactive disease, BILAG score of < 6) were taking hydroxychloroquine alone and all the others were taking one or more immunosuppressants.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Years)</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>BILAG score</th>
<th>Drug treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cyclo mg/mnth</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>F</td>
<td>Caucasian</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>F</td>
<td>A/C</td>
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<td>-</td>
</tr>
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<td>32</td>
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<td>A/C</td>
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</tr>
<tr>
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Table 5.1 continued

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<tr>
<th>Patient</th>
<th>Age (Years)</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>BILAG score</th>
<th>Drug treatments</th>
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<td>Chinese</td>
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<td>Cyclo mg/mnth</td>
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<td>Pred mg/day</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Hydrox mg/day</td>
</tr>
<tr>
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<td>64</td>
<td>F</td>
<td>C’casian</td>
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<td>-</td>
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</tr>
</tbody>
</table>

Key and legend for Table 5.1

F - Female
M - Male
A/C - Afro-Caribbean
C‘casian - Caucasian
mg - milligrammes
a - Used in magnitude experiments, see fig 4.1 and 4.2
The first column numbers patients for the purposes of identification.

5.3 Methods

In chapter 4, a large number (45%) of the patients tested did not produce sufficient levels of anti-dsDNA to be detectable by ELISA and each treatment was tested by three cultures. This resulted in testing a large number of cultures that were negative. To focus time and resources better, it was decided to screen one culture from each treatment set of three for positivity in the anti-dsDNA ELISA, before testing the remaining duplicate cultures. T-cells and monocytes were depleted as described in section 2.2.2, typically approximately 85% of target cells were removed (fig 5.1a-d).

5.4 Results

5.4.1 IgG antibody production

All cultures tested produced measurable quantities of total IgG. In eight patients the B-cells in the depleted and non-depleted cultures were quantified immediately after separation and the cultures set-up to achieve the same final concentration of B-cells, thus allowing comparison of antibody production (section 2.6.2).
Fig 5.1: Cells before and after depletion. Staining methodology is described in section 2.7

(FSC = Forward Scatter; FL-1 = Fluorescence 1 (FITC))

Fig 5.1a: T-cells in lymphocyte gate prior to depletion

FSC-H(1) VS FL1-H(3)

70% of gated PBMCs

30% of gated PBMCs

Fig 5.1b: T-cells in lymphocyte gate after depletion

FSC-H(1) VS FL1-H(3)

8% of gated PBMCs

82% of gated PBMCs

Fig 5.1c: T-cells in lymphocyte gate prior to depletion

FSC-H(1) VS FL1-H(3)

40% of gated PBMCs

60% of gated PBMCs

Fig 5.1d: T-cells in lymphocyte gate after depletion

FSC-H(1) VS FL1-H(3)

4% of gated PBMCs

96% of gated PBMCs

Fig 5.1a-b: Twenty thousand events from a lymphocyte gate are shown. Cells were stained with a non-conjugated, anti-CD3 antibody and a FITC conjugated antibody as described in section 2.7. Fig 5.1a represents the cells prior to depletion and fig 5.1b after depletion of T-cells, see section 2.1.2 for depletion methodology.

Fig 5.1c-d: Fifty thousand events from a gate on total, live PBMCs. Cells were stained with a non-conjugated anti-CD14 antibody and then a FITC conjugated secondary antibody as described in section 2.7. Fig 5.1c represents the cells prior to depletion and fig 5.1d after depletion of monocytes, see section 2.1.2 for depletion methodology.

Eighty-nine percent depletion of T-cells is shown in figs 5.1a and b. Ninety percent depletion of monocytes is shown in 5.1c and d. This level of depletion was typical across experiments.
Table 5.2 shows the average IgG production from the six different types of culture tested (n=8). There were no significant differences between IL-10 treated and spontaneous IgG antibody production in cultures of total PBMC or monocyte depleted PBMC, as measured by the Mann Whitney. However, IL-10 did induce a significant increase in the IgG production from T- depleted PBMC (p = 0.018).

Table 5.2 Mean IgG antibody production from each culture type

<table>
<thead>
<tr>
<th>Culture (n=8, in each case)</th>
<th>IgG production from untreated cultures +/- SE (ng/ml)</th>
<th>IgG production from IL-10 treated cultures +/- SE (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PBMC</td>
<td>325 +/- 88</td>
<td>314 +/- 93</td>
</tr>
<tr>
<td>T-cell depleted</td>
<td>179 +/- 35</td>
<td>211 +/- 33*</td>
</tr>
<tr>
<td>Monocyte depleted</td>
<td>321 +/- 79</td>
<td>347 +/- 85</td>
</tr>
</tbody>
</table>

Table 5.2 After separation, the number of B-cells were quantified and cultures set-up to achieve the same final concentration of B-cells (>200,000 per ½ ml culture). Cells were cultured for three days, then the supernatants harvested and tested by ELISA for IgG level. IL-10 had a statistically significant effect in the T-cell depleted group (p = 0.018, by Mann-Whitney) but in neither of the other groups. IgG level was significantly lower in the T-cell depleted than the other two groups both in treated and untreated states (p < 0.01). There was no significant difference in IgG level between the total PBMC and monocyte depleted groups as measured by Mann Whitney.

5.4.2 Relationship between spontaneous IgG production and disease activity

There were no correlations between disease activity and the production of total IgG or IgG anti-dsDNA from the untreated cultures of total PBMC, or from either of the depletion states (using Spearman Rank correlation). There was a significant correlation between serum anti-dsDNA antibody level and anti-dsDNA production from both the total PBMC and monocyte depleted cultures (r = 0.83; p = 0.021 and r = 0.9; p = 0.006 respectively) [n = 19 in both cases] but not from the T-cell depleted cultures. In addition no correlation between serum anti-dsDNA antibody level and disease activity was seen.
5.4.3 Disease activity, drug treatment and the effect of IL-10 on total IgG

No correlations between disease activity or drug treatment and IL-10 treated IgG antibody production were seen in response to IL-10 in either total PBMC, monocyte depleted or T-cell depleted cultures as measured by regression analysis. In order to focus the analysis on IL-10 induced change and correct for individual antibody production, untreated and treated antibody level were combined as percentage change; however this also failed to show any significant relationships.

5.4.4 Anti-dsDNA antibody production

Forty five percent (19/42) of the patients tested produced measurable quantities of anti-dsDNA antibodies. The raw data and drug treatment of those 19 patients is shown from total PBMC (fig 5.2a and b), T-cell depleted PBMC (fig 5.3a and b) and monocyte depleted PBMC cultures (fig 5.4a and b). Due to the large number of factors present, further analysis with statistical testing will follow.

5.4.5 Effect of T-cell and monocyte depletion on anti-dsDNA antibody production

As described in section 5.4.1, in some cultures the number of B-cells were quantified and equillibrated across cultures. Figs 5.5 and 5.6 show the effects of T-cell and monocyte removal on the magnitude of both spontaneous and IL-10 treated anti-dsDNA antibody production respectively (n = 8 in both cases). Each set of four columns represents one patient, with their disease activity at the time of testing on the x-axis and the antibody production, as a percentage of the positive control, on the y-axis.
Figure 5.2a. PBMCs were aliquoted into 1/2 ml cultures at 2 X 10^6 cells per ml and incubated for three days. Cultures were split into sets of two; one set left untreated ("Untreated") and one set treated with 20ng/ml of IL-10 ("IL-10 treated"). The supernatants were tested for IgG anti-dsDNA level by ELISA. The numbers shown represent the mean OD levels obtained (all cultures were tested on the same ELISA plate). The standard errors are represented by the vertical bars. The x-axis indicates the BILAG score at the time of testing, the patient ID number, the dose of cyclophosphamide (cyclo) in mg/month, methotrexate (MTX) in mg/week, Azathioprine (AZA) in mg/day, prednisolone (pred) in mg/day, hydroxychloroquine (hydrox) in mg/day. Statistical analysis is presented later. This legend also applies to fig 5.2b.
Fig 5.2b Antibody production from total PBMC cultures, Part 2

Fig 5.2b. See legend for fig 5.2a
Fig 5.3a. Experiments were set-up as described in fig 5.2a, except cultures were T-cell depleted using magnetic beads first (see section 2.1.2).
Fig 5.3b Antibody production from T-cell depleted cultures, graph 2

Fig 5.3b. See legend for fig 5.2a
Fig 5.4a Antibody production from monocyte depleted cultures, graph 1

Fig 5.4a. Experiments were set-up as described in fig 5.2a, except cultures were monocyte depleted using magnetic beads first (see section 2.1.2).
Fig 5.4b Antibody production from monocyte depleted cultures, graph 2

Fig 5.4b. See legend for fig 5.4a
Figures 5.5 and 5.6. T-cell and monocyte depletion methodology is described in section 2.2.2. After depletion, the B-cell concentration was determined (see section 2.7.2) and equalised across the cultures. The cells were then set-up in sets of four 1/2 ml cultures containing an equal number of B-cells (> 200,000). One set (four cultures) were undepleted (total) PBMC, one set were T-cell depleted and one set monocyte depleted. Within each set, two cultures were not treated with cytokine and two cultures were treated with 20ng/ml of IL-10. ELISA tested each culture in duplicate, on the same ELISA plate. Mann-Whitney revealed that T-cell depletion reduced spontaneous but not IL-10 treated antibody production (p=0.012). Monocyte depletion made no difference.
Figure 5.6 Effect of monocyte depletion on the magnitude of anti-dsDNA antibody production

Figure 5.6. See legend for fig 5.5
Fig 5.5 shows that T-cell depletion significantly decreased spontaneous, but not IL-10 treated anti-dsDNA antibody production as measured by the Mann Whitney ($p = 0.012$). Fig 5.6 shows that monocyte depletion produced no statistically significant difference in the magnitude of anti-dsDNA antibody production. The effect of IL-10 on change in antibody production is described in section 5.5, as is the impact of drug treatment.

5.5 Disease activity, drug treatment and the effect of IL-10 on anti-dsDNA production

The experiments in this chapter contained many variables: BILAG score (disease activity), untreated antibody production, drug treatment, inter-patient variability and the presence or absence of T-cells or monocytes. In order to test which of these variables played a role in the effect of IL-10 on anti-dsDNA antibody production, the results were analysed by linear regression with the above factors as independent variables and the anti-dsDNA antibody production of the IL-10 treated cultures as the dependent variable. As before (chapter 4) there was an association between mean untreated and mean treated OD. Mean treated OD was also related to BILAG score, but not to any drug treatment. Tables 5.3 and 5.4 present the beta value (standardised co-efficient) and the p value when each of these variables were assessed sequentially in a regression analysis. Table 5.3 presents the data regarding the presence and absence of T-cells and table 5.4 presents the data regarding the presence and absence of monocytes.
Table 5.3 Regression analysis of T-cell involvement in IL-10 induced change in anti-dsDNA antibody production (n = 19)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean untreated antibody level</td>
<td>0.99</td>
<td>0.001</td>
</tr>
<tr>
<td>Global BILAG score</td>
<td>-0.24</td>
<td>0.01</td>
</tr>
<tr>
<td>T-cell: presence/absence</td>
<td>-0.26</td>
<td>0.0014</td>
</tr>
<tr>
<td>Patient</td>
<td>0.82</td>
<td>0.26</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>0.56</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 5.3 Beta value is the standardised co-efficient within a regression function that predicts mean IL-10 treated OD, when that variable is the only independent variable entered.

Table 5.4 Regression analysis of monocyte involvement in IL-10 induced change in anti-dsDNA antibody production (n = 19)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated antibody level</td>
<td>0.92</td>
<td>0.001</td>
</tr>
<tr>
<td>BILAG score</td>
<td>-0.36</td>
<td>0.01</td>
</tr>
<tr>
<td>Monocyte: presence/absence</td>
<td>0.03</td>
<td>0.63</td>
</tr>
<tr>
<td>Patient</td>
<td>0.1</td>
<td>0.12</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>0.65</td>
<td>0.09</td>
</tr>
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</table>

Table 5.4. See legend of table 5.3.

BILAG score and untreated anti-dsDNA antibody production are predictive of the IL-10 treated antibody production; while drug treatment and inter-patient variability (other than patient dependent variation in disease activity, drug treatment and spontaneous antibody production) appeared to be unimportant. These data also demonstrated that T-cells, but not monocytes effected the anti-dsDNA antibody response to IL-10.

Mean untreated antibody level and mean IL-10 treated antibody level are closely related (the associated beta value being almost one), allowing the use of the following formula to express the change in anti-dsDNA antibody production (see
section 2.5). In order to graphically show the relationship between BILAG score and treated OD, untreated and treated OD were combined with the following formula.

This allows the change in antibody production to be seen in terms of the spontaneous level of antibody production, for absolute differences see figs 5.2–5.4.

Mean percentage change in OD = \((\text{Mean treated OD} - \text{Mean untreated OD}) \times 100\)
\[
\text{Mean untreated OD}
\]

**Figure 5.7** Relationship between disease activity and IL-10 Induced change in anti-dsDNA antibody production

**Figure 5.7.** Antibody production is represented by the percentage change induced by IL-10 (20ng/ml), formula on previous page. Each point is the mean difference between two pairs of cultures each tested in duplicate on the same plate. Each culture contained \(2 \times 10^6\) total PBMC per ml in \(1/2\) ml samples and was cultured for three days. Spearman Rank correlation revealed a statistically significant relationship between disease activity and IL-10 induced change in antibody production \((r = -0.52, p = 0.022, n = 19)\).
Fig 5.7 plots disease activity against the IL-10 induced change in anti-dsDNA antibody production from total PBMC (percentage change being calculated with the formula presented on the previous page). Change in anti-dsDNA antibody production correlated with disease activity such that patients with inactive disease increased their antibody production while those with active disease decreased their antibody production ($r = -0.52, p = 0.022$); replicating the results in chapter 4.

### 5.5.1 Effect of T-cell and monocyte depletion on IL-10 induced change in anti-dsDNA antibody production

Figure 5.8 shows the effect of T-cell depletion on the anti-dsDNA antibody response to IL-10. Each pair of columns represents one patient, with the disease activity at the time of testing on the x-axis and the mean percentage change in anti-dsDNA production on the y-axis. Fig 5.8 shows that T-cell depletion (greater than 85% removal was achieved, see fig 5.1a-d) reverses the inhibitory effects of IL-10 at high disease activities, but does not effect the augmenting effects of IL-10 at low disease activities. Therefore, in the absence of T-cells, IL-10 caused a consistent increase in antibody production regardless of disease activity. These observations reflect the statistical analysis summarised in table 5.3.

Figure 5.9 shows the effect of monocyte depletion on the anti-dsDNA antibody response to IL-10 and is set out identically to figure 5.8. Monocyte depletion (greater than 85% removal was achieved, see fig 5.1a-d) did not have a statistically significant effect (see table 5.4). Monocyte removal did not alter the direction of change (increase or decrease) in response to IL-10. The antibody production of some patients (3, 7, 13, 14, and 15) was altered by monocyte depletion (as measured by paired T-test, $p < 0.05$) but no consistent pattern could be found, reflecting the failure to find any statistical relationship.
Figures 5.8 and 5.9. T-cell and monocyte depletion methodology is described in section 2.2.2. After depletion, the cells were set-up in sets of four 1/2 ml cultures. One set (four cultures) were undepleted (total) PBMCs, one set were T-cell depleted and one set monocyte depleted. Within each set, two cultures were not treated with cytokine and two cultures were treated with 20ng/ml of IL-10. Each culture was tested in duplicate by ELISA, on the same ELISA plate. The y-axes present the percentage change induced by IL-10 (20ng/ml), formula on previous page. Each point is the mean difference between one untreated and one IL-10 treated culture within one set. Each culture contained 2 X 10^6 total PBMCs per ml in 1/2 ml samples and was cultured for three days. See tables 5.3 and 5.4 for statistical analysis.
Fig 5.9 Effect of monocyte depletion on the percentage change in antibody production in response to IL-10

Fig 5.9. See legend for fig 5.8
5.6 Discussion

5.6.1 Summary of results

In this thesis the results of Linker-Israeli et al. (1990) have been confirmed suggesting that T-cell removal decreases total IgG and IgG anti-dsDNA antibody production. It has also been demonstrated that monocyte depletion does not significantly alter antibody production. IL-10 did not significantly alter IgG antibody production in the absence of monocytes but caused a significant increase in the absence of T-cells (replicating the results of Llorente et al. 1995). As shown in chapter four, IL-10 did not significantly alter the IgG production from total PBMC.

A proportion of patients with SLE produced sufficient anti-dsDNA antibodies to be measured by ELISA. In the total PBMC cultures, IL-10 caused a change in anti-dsDNA antibody production that correlated with disease activity such that patients with inactive disease increased their antibody production while those with active disease decreased their antibody production ($r = -0.52; p = 0.022$); replicating the results described in chapter four. The repeatability of this finding argues against it being a statistical aberration, while the failure to find a relationship with hydroxychloroquine treatment suggests that it may have been an anomalous result.

T-cell depletion reversed the aforementioned inhibitory effects of IL-10 at high disease activities while not affecting the augmenting effect of IL-10 in patients with inactive disease. Monocyte depletion did not have a statistically significant effect on anti-dsDNA antibody production.
5.6.2 Criticism of the experiments in chapter 5

The same criticisms detailed in section 4.5.5 are applicable to this chapter. Additionally, only one technique for T-cell and monocyte depletion was employed. Iron filings or Petri dish adherence could have been tested for monocyte removal and sheep cell rosetting could have been attempted to deplete for T-cells. No estimation of cell activation was undertaken after depletion. The depletion technique may have altered the activation state of the remaining cells, which would effect their response to IL-10.

An insufficient number of patients were tested to analyse the number of variables present and those associations found were weak. Larger numbers of patients would be needed to assess whether the associations found were valid.

The data was finally presented as mean percentage change. Absolute difference could be seen from the raw data and the regression analysis. However, percentage change controls for the variation in magnitude of antibody production between patients; therefore focussing in on the possible effect of IL-10 and therefore possibly magnifying any effect that it has.

5.6.3 Effect of IL-10 on T-cell depleted cultures

5.6.3a T-cells and total IgG production

The increase in total IgG antibody production from T-cell depleted cultures in response to IL-10 at 20ng/ml is consistent with the findings of Llorente et al. (1995). In contrast, in the presence of T-cells, IL-10 did not significantly alter IgG antibody production. Linker-Israeli et al. (1990) demonstrated that T-cell removal
decreased *in vitro* IgG antibody production, a result replicated in chapter 5. These observations suggest that T-cells contribute to the *in vitro* hyperproduction of IgG found in patients with SLE. It is therefore possible that in the presence of T-cells the spontaneous IgG production is so high that it masks any effect of the IL-10. Another possibility is that T-cells maximally stimulate B-cells through the pathway utilised by IL-10. This is consistent with the observation that anti-IL-10 decreases spontaneous antibody production (al-Janadi *et al.* 1996). However, the level of spontaneous IL-10 production by T-cells was not quantified in this study. Nor were the comparative effects of IL-10 inhibition and T-cell removal. Combined, these omissions make any further discussion as to the interaction of T-cells and IL-10 on antibody production impossible.

### 5.6.3b T-cells and anti-dsDNA antibody production

T-cell removal also decreased anti-dsDNA antibody production (Linker-Israeli *et al.* (1990) and chapter 5). Depletion experiments suggested that T-cells are responsible for the inhibitory effect of IL-10 at high disease activities (Fig 5.7). Results of linear regression analysis were compatible with this suggestion (beta = -0.26; p = 0.0014).

Interestingly, T-cell depletion only appeared to effect the cells from patients with active disease, but not those with inactive disease. Georgescue *et al.* (1997) have shown that IL-10 at 20ng/ml induces an increase in T-cell apoptosis from the PBMC of patients with active SLE, while not affecting those with inactive disease (as measured by SLEDAI). This increased rate in T-cell apoptosis could be inhibited by fas blockade, suggesting that this was an example of activation inducing cell death rather than a deficit in clearance.
clearance has been implicated in the increased in vitro apoptotic count in cultures of PBMC from patients with SLE (Herrmann et al. 1998). The number of active, peripheral T-cells has been reported to correlate with disease activity as measured by SLEDAI (Spronk et al. 1996). This observation might explain their increased sensitivity to activation induced apoptosis.

One possibility therefore is that an increase in T-cell death might mimic the effects of T-cell depletion and therefore explain the inhibitory effects of IL-10 on the anti-dsDNA antibody production from patients with active but not inactive SLE. In addition, an increase in the level of apoptotic fragments in the supernatants might complex the anti-dsDNA antibodies, thus selectively decreasing their apparent level.

This may explain the observation seen in chapters 4 and 5, that in active disease IL-10 cannot change in vitro total IgG production from total PBMC. As in the presence of T-cells in active disease IL-10 operates two conflicting mechanisms in vitro. However, it would not explain the lack of response in patients with inactive disease. The fact that this study is not suitably comprehensive to suggest that this lack of response may be due to insufficient prestimulation of B-cells has been discussed (See section 4.5.4).

Though hydroxychloroquine treatment was not found to be predictive of IL-10 induced treatment in this cohort, in chapter 4 it was found that patients taking it were more likely to increase their antibody production in response to IL-10 (see section 4.5.5). The purported ability of hydroxychloroquine to decrease activation induced cell death (Lui et al. 2001) would predict that it might block the decrease in antibody production induced by IL-10 (In the event of the above hypothesis being true).
The selective loss of activated T-cells from the culture may result in a significant decrease in the available CD40 ligand. Experiments in healthy controls are divided as to what effect the removal of CD40 ligand will have. Some studies show that CD40 stimulation is required for IL-10 to have any measurable effect on antibody production (Rousset et al. 1995). While other studies show that the removal of CD40 stimulation from CD40 pre-stimulated cells signals plasma cell differentiation (Liu and Banchereau 1997). The combination of IL-10 and CD40 stimulation favouring memory cell differentiation as opposed to plasma cell differentiation (Liu and Banchereau 1997). This observation would predict that IL-10 might decrease antibody production in patients with active disease, independent of the effect of IL-10 on T-cell apoptosis. However, the absence of staining for CD40 ligand or plasma / memory cell markers makes any further discussion of this possibility impossible.

Additionally, to consider CD40 to the exclusion of all other known important signalling mechanisms (such as OX40) not to mention those mechanisms not yet described is a clear oversimplification. This concern reiterates that the study of complex mixed cell cultures in a complex disease requires a level of preliminary experimentation not fully undertaken in this thesis.

5.6.4 Effect of IL-10 on monocyte depleted cultures

Monocyte depletion did not alter the magnitude of \textit{in vitro} antibody production, despite being the major source of \textit{in vitro} IL-10 production from SLE PBMC (Llorente et al. 1993). This would suggest that other cell types can also produce IL-10 or substitute another factor, or that endogenous IL-10 is not required for \textit{in vitro} antibody production. In addition monocyte depletion did not alter the effect
of IL-10 on antibody production. These results would suggest that either, monocytes are not involved with in vitro antibody production, or that monocyte removal has two equal and opposing effects.

There is no suggestion in the literature that monocytes could effect B-cells directly, however monocytes can effect T-cells and, as has been discussed above, T-cells can effect in vitro antibody production. It is thought that the monocytes from SLE patients tend to inhibit the activation of SLE T-cells (Horwitz et al. 1997) and IL-10 has been shown capable of inducing this ability in the monocytes of HC (Ding et al. 1992, Lauwerys et al. 2000).

Therefore monocyte removal might be expected to magnify any activating signal and block at least some of the inhibitory actions of IL-10. The data shown (fig 5.9) suggests that monocyte depletion does effect the antibody production from some patients (patients 3, 7, 13, 14 and 15), but does not have a consistent effect. Clearly other variables are involved, perhaps differences in the activation state of the cells at time zero.

5.6.5 The effect of IL-10 on patients with inactive disease

The observation that neither T-cell nor monocyte removal appeared to block the IL-10 induced change in the anti-dsDNA antibody production from patients with inactive disease consistently, suggests that IL-10 may be acting through another cell type (perhaps NK cells) or having a direct effect on B-cells. IL-10 can act directly on pre-activated B-cells from HC to increase antibody production (De Waal Malefyt et al. 1993). Patients with both inactive and active SLE have a higher than normal number of activated B-cells in their periphery
Together these data are compatible with the suggestion that IL-10 may be acting directly on the B-cells of patients with inactive disease.

5.6.6 Conclusion

In conclusion it appears that T-cells are involved with the \textit{in vitro} anti-dsDNA antibody production of patients with SLE and the response of that antibody production to IL-10, while monocytes are probably not. Therefore hypothesis 1 (T-cell depletion will reverse the inhibitory effects of IL-10 on the anti-dsDNA antibody production from the PBMC of patients with SLE who have higher disease activity) is provisionally accepted, while hypothesis 2 (Monocyte depletion will abrogate the effect of IL-10 on anti-dsDNA antibody production from the PBMC of patients with SLE) is provisionally rejected.
Chapter 6
The effect of IL-10 on T-cell apoptosis and anti-dsDNA antibody production

Hypothesis: IL-10 induced T-cell apoptosis is a stronger predictor of IL-10 treated anti-dsDNA antibody level than BILAG score.

6.1 Introduction
Chapter 4 showed a correlation between disease activity and IL-10 induced change in anti-dsDNA antibody production, such that patients with inactive disease increased their antibody production while patients with active disease decreased their antibody production. Chapter 5 showed that this dichotomy may be due to T-cells, as T-cell depletion reversed the inhibitory effect of IL-10 in patients with active disease while largely not affecting the antibody production from patients with inactive disease.

Georgescue et al. (1997) have shown that IL-10 treatment causes the T-cells from patients with active SLE, but not inactive SLE, to increase their apoptotic rate. This might result in IL-10 inhibiting the anti-dsDNA antibody production from the PBMC of patients with active SLE through two mechanisms. Firstly, T-cell depletion has been shown to reduce anti-dsDNA antibody production (Linker Israeli et al. 1990 and Chapter 4). Secondly, increased T-cell apoptotic rate may increase cell debris within the supernatant. This effect could selectively decrease the apparent anti-dsDNA antibody level by complexing the antibodies and therefore preventing them from binding to the ELISA plate.
Total PBMC were cultured with or without IL-10, the supernatants tested for anti-dsDNA antibody production and the cells tested for their level of T-cell apoptosis by FACs analysis (see section 2.6).

It was shown that the IL-10 induced change in T-cell apoptosis is more predictive for the IL-10 induced change in anti-dsDNA antibody production than disease activity. Thus the underlying cause of the association between disease activity and IL-10 induced change in anti-dsDNA antibody production could be the effect of IL-10 on T-cell apoptosis.

6.2 Patients and methods

Fourteen of the patients with SLE tested, produced a sufficient quantity of anti-dsDNA to be measurable by ELISA. Each of the patients with SLE studied met four or more of the revised classification criteria for SLE (Tan et al. 1982). Patients were selected at random from amongst those known to have currently raised serum anti-dsDNA antibody levels which were re-tested at the time of visit to the clinic. Disease activity was assessed using the British Isles Lupus Assessment Group (BILAG) index (see Introduction 1.1.3 and appendix). All of the fourteen patients were female: one Asian, eleven Caucasians and two Afro-Caribbeans; the mean age of this group was 34 years (range 21 - 46). (See table 6.1 for further details).

<table>
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<tr>
<th>Patient</th>
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<th>Pred mg/day</th>
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Key and legend for Table 6.1

A/C – Afro-Caribbean
C’casian – Caucasian
mth – month

MTX – Methotrexate
AZA – Azathioprine
Hydrox – Hydroxychloroquine

In this chapter the relationship between disease activity and the effect of IL-10 on the change in antibody production and T-cell apoptosis is of primary importance. Therefore the sections on spontaneous antibody production that were presented in the previous two chapters will be omitted here. The experimental conditions described in chapters 4 and 5 were applied in chapter 6. After the supernatants had been harvested on day three, the extent of T-cell apoptosis was quantitated. Cells from the three identically treated cultures were diluted in warmed RPMI and washed once (see section 2.2.1). The cells were then stained for T-cell and apoptotic markers as described in section 2.6.3.

6.3 Results

Figure 6.1 shows the staining of one patient’s untreated and IL-10 treated PBMC as an example. Figures 6.2a and b show the untreated and IL-10 treated antibody levels and figs 6.3a and b show the level of T-cell apoptosis in the untreated and IL-10 treated cultures. Due to the number of factors presented, statistical analysis will be shown on those graphs that best demonstrate the significant relationships found.
Figure 6.1. FACscan analysis of T-cell apoptosis in untreated and IL-10 treated PBMC cultures

PBMCs were cultured at $2 \times 10^6$ cells per ml in 0.5 ml samples. Cultures were divided into sets of three, one set was left untreated and one set was treated with 20ng/ml of IL-10. After three days, PBMCs were stained for annexin (FL-1) and CD3 (FL-2). Figure 6.1 is an example of the staining achieved on the CD3+ gated PBMCs. 6.1a is untreated and 6.1b is from the IL-10 treated culture. 20% of gated PBMC were annexin positive in 6.1a and 35% in 6.1b. 20,000 events were acquired in each case.
Figure 6.2a. Antibody production, graph 1

Figure 6.2a. PBMC were aliquoted into 1/2 ml cultures at 2 × 10^6 cells per ml and incubated for three days. Cultures were split into sets of three; one set left untreated (“Untreated”) and one set treated with 20ng/ml of IL-10 (“Treated”). The supernatants were tested for anti-dsDNA level by ELISA. The numbers shown represent the mean OD levels obtained (all cultures were tested on the same ELISA plate). The standard errors are represented by the vertical bars. The x-axis indicates the BILAG score at the time of testing, the patient ID number, the dose of cyclophosphamide (Cyclo) in mg/month, methotrexate (MTX) in mg/week, Azathioprine (AZA) in mg/day, prednisolone (Pred) in mg/day, hydroxychloroquine (Hydrox) in mg/day. Statistical analysis is presented later.
Figure 6.2b Antibody production, graph 2

Figure 6.2b. See legend for figure 6.2a
Figure 6.3a T-cell apoptosis, graph 1

Figure 6.3a. Experiments were set-up as described in the legend for fig 6.1. After the supernatants had been harvested for ELISA, cells were diluted in warmed RPMI, washed once (see section 2.2.1) and then stained with annexin and anti-CD3 to mark apoptotic cells and T-cells respectively (see section 2.2.11). Mean percentage of apoptotic T-cells are shown in fig 6.2a and b. Statistical analysis is shown later.
Figure 6.3b T-cell apoptosis, graph 2

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<th>Pred</th>
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untreated □  IL-10 treated

Fig 6.3b. See legend for fig 6.3a
6.3.1 T-cell apoptosis and disease activity

The relationship between disease activity and T-cells apoptosis is shown in figs 6.4a and 6.4b. The percentage of annexin positive (apoptotic) T-cells is graphed against BILAG score in the untreated (6.4a) and IL-10 treated cultures (6.4b). There is no relationship between disease activity and T-cell apoptosis in the untreated cultures, however, T-cell apoptosis did correlate with disease activity in the IL-10 treated cultures ($r = 0.74$, $p = 0.03$, $n = 14$) as measured by Spearman rank correlation.

6.3.2 IL-10 induced change in anti-dsDNA production and disease activity

To demonstrate that T-cell apoptosis is more predictive of IL-10 induced change in anti-dsDNA antibodies than BILAG score it must be shown that BILAG score is predictive in this cohort. Raw data is presented in figs 6.2a and b, however, to show the relationship graphically in an optimal way mean percentage change in OD is compared to BILAG score (fig 6.5). [Untreated OD predicts treated OD, ($r = 0.9$) see section 2.5]. Spearman rank showed a statistically significant correlation between these two parameters ($r = -0.559$, $p = 0.041$, $n = 14$). The power of BILAG score to directly predict IL-10 treated OD is presented in table 6.2.
Duplicate cultures were set-up at $2 \times 10^6$ cells per ml in ½ ml cultures and left untreated. On day three supernatants were taken, the remaining PBMCs were diluted in warm RPMI and washed once (see table 2.2.1). Cells were stained for apoptotic cells (annexin) and T-cells (CD3). Spearman rank revealed no statistically significant correlation between disease activity and percentage of apoptotic T-cells in untreated cultures.
Figure 6.4b Relationship between disease activity and the percentage of T-cell apoptosis in total PBMC cultures treated with IL-10

Figure 6.4b. Duplicate cultures were set-up at $2 \times 10^6$ cells per ml in $\frac{1}{2}$ ml cultures and treated with 20ng/ml of IL-10. On day three supernatants were taken, the remaining PBMCs were diluted in warm RPMI and washed once (see table 2.2.1). Cells were stained for apoptotic cells (annexin) and T-cells (CD3). Spearman rank revealed a statistically significant correlation between disease activity and percentage of apoptotic T-cells in IL-10 treated cultures ($r = 0.74$, $p = 0.03$, $n = 14$).
Figure 6.5. Antibody production is represented by the percentage change induced by IL-10 (20ng/ml), see section 2.5. Each point is the mean difference between two pairs of cultures each tested in duplicate on the same plate. Each culture contained $2 \times 10^6$ total PBMC per ml in $1/2$ ml samples and was cultured for three days. Spearman Rank correlation revealed a statistically significant relationship between disease activity and IL-10 induced change in antibody production ($r = -0.55, p = 0.041, n = 14$).
6.3.3 The relationship between apoptosis and anti-dsDNA antibody production

The experiments in this chapter contained many variables: BILAG score (disease activity), untreated antibody production, drug treatment, inter-patient variation, spontaneous T-cell apoptosis and IL-10 induced T-cell apoptosis. Multiple linear regression analysis was used to discover which, if any, of these variables predicted IL-10 treated anti-dsDNA level. A stepwise analysis was undertaken. This analysis instructs the programme to try each independent variable, in each combination, in order to find the combination of independent variables that can best explain the dependent variable. Table 6.2 shows the beta value (standardised co-efficient within the multiple linear regression) and the associated, corrected p value for the most predictive combination of variables.

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<th>Variable</th>
<th>Beta</th>
<th>p-value</th>
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<td>Untreated antibody level</td>
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<td>BILAG score</td>
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<tr>
<td>Overall regression constant</td>
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</table>

Table 6.2. Beta value is the standardised co-efficient within the multiple linear regression, p value the chance of the association found being due to chance.

Neither IL-10 treated nor untreated T-cell apoptosis could replace BILAG score in the most predictive combination of independent variables. These results suggest that the underlying reason for the association between BILAG score and IL-10 treated antibody level is not T-cell apoptosis. However, if the analysis is focussed on the effect of IL-10 on T-cell apoptosis by entering difference in T-cell apoptosis associated with IL-10 treatment (mean percentage of T-cell apoptosis in
IL-10 treated cultures minus that in untreated cultures); then T-cell apoptosis becomes more predictive, see table 6.3.

Table 6.3 Regression analysis of the inter-relationship between T-cell apoptosis and anti-dsDNA antibody production in response to IL-10

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<tr>
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<td>0.023</td>
</tr>
<tr>
<td>Overall regression constant</td>
<td>17.9</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Table 6.3. Beta value is the standardised co-efficient within the multiple linear regression, p value the chance of the association found being due to chance.

Drug treatment, inter-patient variation (other than patient dependent variation in disease activity, spontaneous antibody production and drug treatment) and the spontaneous rate of T-cell apoptosis and IL-10 induced apoptosis alone are not as predictive.

The inclusion of IL-10 induced difference in apoptosis and BILAG score together improved the overall regression and rendered the beta value for BILAG score non-significant (p > 0.05). This result suggests that the IL-10 induced difference in T-cell apoptosis can replace disease activity.

6.3.4 Demonstration of relationship between T-cell apoptosis and anti-dsDNA

Untreated antibody production and IL-10 induced difference in T-cell apoptosis were most predictive of IL-10 induced change in anti-dsDNA production. To demonstrate this on a graph, untreated and IL-10 treated anti-dsDNA antibody level is combined using percentage change in anti-dsDNA antibody production (see fig 6.6). As there is a strong relationship between untreated and IL-10 treated antibody level this is acceptable (see section 2.5).
Figure 6.6. PBMC were aliquoted into $\frac{1}{2}$ ml cultures at $2 \times 10^6$ cells per ml and incubated for three days. Cultures were split into sets of three; one set left untreated (“Untreated”) and one set treated with 20ng/ml of IL-10 (IL-10 treated). The supernatants were tested for anti-dsDNA level by ELISA. The x-axis presents the mean difference in T-cell apoptosis between the two sets of cultures. The y-axis presents the mean percentage difference between two sets of cultures (see section 2.5) each tested in duplicate on the same plate. Spearman rank revealed a statistically significant correlation between the two variables ($r = -0.815$, $p < 0.01$, $n = 14$).
6.4 Discussion

6.4.1 Summary of results

As in chapters 4 and 5, it was found that IL-10 caused a change in anti-dsDNA antibody production that correlated with disease activity, such that it increased antibody production in patients with inactive disease but decreased it in patients with active disease (table 6.2, fig 6.5). These data showed that T-cell apoptosis correlated positively with disease activity in IL-10 but not untreated cultures (Figs 6.4a and b) consistent with the findings of Georgescue et al. (1997). It is interesting to note that there was no correlation between disease activity and the rate of T-cell apoptosis (fig 6.4a). As it is suggested that T-cell activation is higher in active disease (Spronk et al. 1996) which would imply a higher rate of activation induced cell death. Activation state and fas activity was not assessed in these studies, however this observation deserves further elucidation.

In this chapter, IL-10 induced T-cell apoptosis could substitute for disease activity with a greater predictive power for the effect of IL-10 on anti-dsDNA antibody production (Table 6.3 and Fig 6.6). This observation suggested that the initial correlation found between disease activity and IL-10 induced change in anti-dsDNA antibody production was, at least partially, due to the increased sensitivity of the T-cells from patients with active disease to the pro-apoptotic signals of IL-10.

6.4.2 Criticism of the experiments in chapter 6

In addition to the criticisms stated in chapter 4 (see section 4.5.2) there are a number of other concerns. Only one method for estimating T-cell apoptosis was
employed, clearly these data would be more convincing if corroborated by further techniques.

Fourteen patients are not sufficient to fully analyse the number of variables present and those associations found were weak. Therefore some doubt must be expressed regarding all conclusions made. In addition, a mathematical derivative of T-cell apoptosis was required to find a significant relationship. A direct relationship would have been more acceptable.

6.4.3 Other possible underlying causes, besides T-cell apoptosis

As discussed in the Introduction (see section 1.10.5), the T-cells responsible for anti-dsDNA antibody production are thought to be Th1 (Voll et al. 1997) and IL-10 has been shown to inhibit the cytokine production of Th1 cells (Mosmann and Moore 1991). The T-cells from patients with active disease appear to be preferentially sensitive to IL-10. It might therefore be possible that IL-10 treatment is selectively either killing, inhibiting or changing the cytokine profile of the anti-nucleosome T-cells from the patients with active disease and therefore decreasing anti-dsDNA antibody production. It is also possible that IL-10 may be effecting another cell type, for example NK cells, which have been shown to play a role in the in vitro antibody production of PBMC from patients with SLE (Linker Israeli et al. 1990). It is also possible that in increasing the level of T-cell apoptosis a significant number of anti-dsDNA are being sequestered resulting in the observed decrease in antibody. This would render this observation a purely in vitro phenomenon of little interest to the in vivo situation.
6.4.4 Possible clinical significance

Llorente et al (2000) administered an anti-IL-10 monoclonal antibody to six patients with SLE. While this treatment was effective in reducing disease activity, it only decreased anti-dsDNA antibody production in one patient (with inactive disease, as calculated by a global score system, SLEDAI). The serum anti-dsDNA antibody titres of the other patients remained largely unchanged. The existing literature would have predicted that anti-IL-10 treatment would decrease anti-dsDNA antibody production regardless of disease activity (Llorente et al. 1995, Ishida et al. 1994, al-Janadi-M et al. 1996). This thesis would predict that anti-IL-10 would decrease anti-dsDNA antibody production from patients with inactive disease (if it was raised) while increasing it from patients with active disease.

Clearly, an in vitro model can only supply information on the state of the immune system at a single moment, whereas in vivo, the immune system will be under constant, dynamic change. The model in this thesis demonstrates that IL-10 can have a mixed effect on anti-dsDNA antibody production, perhaps explaining the failure of anti-IL-10 to have a significant overall effect on anti-dsDNA production in vivo.

6.5 Conclusion

It appears that the inhibitory effect of IL-10 on anti-dsDNA antibody production at high disease activities is, at least partly, due to its ability to increase T-cell apoptosis; thus the hypothesis of this chapter is not rejected. However, without further elucidation of the mechanism the possibility that this is an in vitro phenomenon cannot be discounted. In addition, it may be that any immune stimulant would have produced an identical result as no such control was used.
Chapter 7.0

General discussion

7.1 Spontaneous production of anti dsDNA *in vitro* by blood mononuclear cells from SLE patients; relationship to disease activity and other parameters

7.1.1 Cultures of unfractionated blood peripheral blood mononuclear cells (PBMC)

In initial studies using whole PBMC, spontaneous production of IgG anti-dsDNA antibodies *in vitro* were detected in around 50% of patients with SLE. None of the controls showed detectable levels. Total IgG was detectable in all patients' PBMC cultures and all 16 healthy controls. The reason why in only some patients anti-dsDNA antibodies were detectable *in vitro* is unclear but could include lack of effective T cell help, lower levels of anti dsDNA specific B cells or insufficient B-cell activation at time zero. It was interesting that in cross-sectional studies the spontaneous production of anti-dsDNA *in vitro* did correlate with serum anti-dsDNA titre but not with any measure of disease activity. However, in the longitudinal study there was a correlation between spontaneous anti-dsDNA antibody production, serum anti-dsDNA titre and disease activity as measured by BILAG converted to a global scale. The failure to find a correlation between anti-dsDNA antibody level and disease activity in the cross-sectional study is probably due to the wide variation in anti-dsDNA antibody titre between patients, a problem that was overcome by testing the patients longitudinally as it controlled for inter-patient variability. This correlation between serum anti-dsDNA titre and spontaneous anti-dsDNA antibody production also suggests that the peripheral
blood B cell response mirrors that of the overall lymphoid tissues. In those patients that were not producing appreciable amounts of anti-dsDNA \textit{in vitro} at the time of testing, the antigen specific B cells were possibly localised in the lymphoid tissues such as bone marrow or spleen rather than the peripheral blood.

The level of spontaneous IgG and IgG anti-dsDNA antibody production by SLE lymphocytes depleted of T cells were decreased in cultures seeded with an equal number of B-cells. This T cell dependence was also shown by Linker-Israeli \textit{et al.} (1990). In these studies, monocyte depletion did not significantly alter spontaneous IgG or IgG anti-dsDNA antibody production.

### 7.2 Effect of IL-10 on \textit{in vitro} anti dsDNA production by SLE blood mononuclear cells: mechanisms

Contrary to expectation, in this study IL-10 not only increased anti-dsDNA antibody production (in patients with inactive SLE) but also decreased anti-dsDNA antibody production (in patients with active disease). This was in contrast to the data on total IgG levels which were unchanged; whereas the studies of Llorente \textit{et al} (1995) found a consistent increase. The possible reasons for the differences seen between these studies will be discussed later.

#### 7.2.1 The role of T cells in the modulation of antibody production by IL-10

In order to determine the mechanisms by which IL-10 was inducing changes in anti-dsDNA production, experiments were carried out on the direct effect of IL-10 on T-cell depleted blood mononuclear cells. In the absence of T-cells IL-10 consistently increased anti-dsDNA antibody production regardless of disease
activity. T-cell depletion did not appear to alter the response to IL-10 of patients with inactive disease, who increased their antibody production with and without T-cells, but reversed its effect in patients with active disease who would otherwise tend to decrease their antibody production. This observation suggested that IL-10 effected the T-cells from patients with active disease in a way that decrease, or removed help for, antibody production but not the T-cells from patients with inactive disease. Thus IL-10 sensitivity may change with disease activity.

Many variables could effect the sensitivity of cells to IL-10, including the magnitude of change in the IL-10 concentration (from \textit{in vivo} to \textit{in vitro}) and other immune signals experienced by the cells both in culture and while they were \textit{in vivo}.

It is known that a greater proportion of peripheral T-cells express an active phenotype in patients with active SLE than inactive SLE (Spronk et al. 1995). It was proposed that the T-cells in active SLE might be IL-10 receptor positive while they are negative in inactive disease. However, studies to map the distribution of the IL-10 receptor on the PBMC from patients with SLE did not achieve consistent detection within the time available. The concentration of IL-10 in supernatants and serum was not quantitated in this thesis. Clearly all factors could not be assessed, however these would be of interest in future study.

7.2.2 Role of monocytes in modulation of anti dsDNA antibody by IL-10

If IL-10 is responsible for increasing anti-dsDNA antibody production, then monocyte depletion would be expected to decrease antibody production as they are thought to be the main source of IL-10 production \textit{in vitro} (Llorente \textit{et al.} 1993).
Monocyte depletion did not significantly alter the effect of IL-10 on anti-dsDNA antibody production. This observation suggested that monocytes were not involved with antibody production and are not involved with the effect of IL-10 on antibody production.

7.2.3 T cells and apoptosis mediated by IL-10

It appeared that T-cells but not monocytes were responsible for the inhibitory effect of IL-10 on anti-dsDNA antibody production. It seemed likely that a T-cell response to IL-10 that varied with disease activity was responsible for the results seen in chapter 4. Georgescue et al (1997) had shown that the T-cells from patients with active but not inactive disease (as defined by SLEDAI) increased their apoptotic rate in response to IL-10. This result was replicated in chapter 6, moreover the difference in T-cell apoptosis induced by IL-10 was more predictive of the change in anti-dsDNA antibody production in response to IL-10 than disease activity. Thus T-cell apoptosis might be (partly) responsible for the observation that the effect of IL-10 on anti-dsDNA antibody production varied with disease activity.

7.3 Future studies

7.3.1 Improvements

To decrease the possible number of interactions, a simpler culture system would be beneficial. Rather than starting with a complex system (total PBMC) from which individual cell types are removed (as done here), it may have been easier to interpret the results from a simple system containing only one cell type. As stated
Repeatedly in chapters 4 to 6, more preliminary experiments were required to allow comparison with the published literature.

Firstly, a pure population of B-cells (obtained by a depletion methodology similar to that employed in chapter 5) could have been used. Additional experiments would have to include measurement of serum IL-10 and spontaneous IL-10 production. Therefore change in IL-10 concentration could be estimated and graphed against effect. Ex vivo activation state should be measured and tested at different time points throughout the culture period. From such experiments it could be seen when IL-10 is released and when it has the maximal effect on antibody production. This could be compared to identical cultures in HC. It is likely that some pre-activation would be required to bring HC activation state to that of "resting" activation state in lupus B-cells. CD40 and IL-2 levels should also be estimated ex vivo and at different time points within the culture as these molecules are known to interact with the effect of IL-10 on antibody production. The effect of CD40 and IL-2 should be measured, both by addition and inhibition experiments.

From such experiments it may be possible to test if the control of anti-dsDNA production is different from that of total IgG. It would also have been useful to compare the effect on an antigen that both HC and SLE patients PBMC may respond to, for example tetanus toxoid post immunisation. Therefore antigen specific responses could be tested and compared to HC. In addition the level of antigen (tetanus toxoid) within the culture could be controlled, whereas DNA could not.

After these experiments the effect of IL-2, CD40 and antigen on IL-10 induced change in antibody production would be known. T-cells could then be
characterised in terms of their CD40 expression and IL-2 production. From there the effect of inclusion of T-cells in these cultures could be predicted. Therefore allowing a hypothesis driven assessment of the role of T-cells.

In addition the percentage of plasma cells at different time points could be quantified. Then the antibody level could be related to the number of plasma cells.

7.3.2 How does IL-10 induced T-cell apoptosis reduce anti-dsDNA antibody production?

The discussion within this section assumes agreement with the findings of this thesis. As has been discussed, the relationships found in this thesis may be due to chance, but the main finding of a correlation between disease activity and IL-10 associated change in anti-dsDNA antibody production was repeated three times.

As described in chapter 5, T-cell apoptosis appears, at least partially, to explain the ability of IL-10 to reduce anti-dsDNA antibody production from patients with active disease. The precise mechanism by which T-cell apoptosis reduces anti-dsDNA antibody production was not described. Many possible mechanisms exist, which may be working in parallel. Therefore, a comprehensive answer to this question requires relative quantification of each mechanism likely to be involved.

7.3.2a Apoptotic fragments sequestering anti-dsDNA antibodies and decreasing the apparent level of anti-dsDNA antibodies as measured by ELISA.

The quantification of this effect on apparent anti-dsDNA antibody level could be done directly or indirectly. If done directly, the quantity of antibodies in DNA containing immune complexes could be estimated, using the technique described
by Emlen et al. (1988), this approach is likely to be technically challenging due to the low levels of antibody involved. If done indirectly, the number of apoptotic T-cells could be calculated and the effect of that number of apoptotic T-cells on the apparent level of a quantitated solution of anti-dsDNA antibodies measured.

7.3.2b Could IL-10 change the Th1: Th2: Th3: Th0 ratio of the T-cells in cultured PBMC?

As described in section 1.10, IL-10 has been shown to be capable of altering the Th response type to an antigen (Akdis and Blaser 1998). It may be that IL-10 is causing apoptosis preferentially in a specific Th subtype, and/or actually altering the cytokine secretion pattern of certain T-cells. PBMC could be separated from blood and split into three equal groups, one group cultured for three days in plain medium, one group cultured for three days in IL-10 (20ng/ml) and one group stained for FACs analysis \textit{ex vivo}. Cells would be stained for CD3 (T-cell marker), annexin (apoptosis marker) and intracellularly stained for a cytokine (IFN\textgamma for Th1, IL-4 for Th2 and TGF\beta for Th3). Six colour staining is now possible on some FACs machines. It would also be important to undertake a time line experiment to test Th profiles during the culture period.

7.3.3 Could IL-10 effect the number of plasma cells in culture?

As described in section 1.5, IL-10 can effect the differentiation of B-cells into plasma or memory B-cells. It may-be that exogenous IL-10, in addition to the signalling milieu of active disease, inhibited plasma cell differentiation/survival whereas IL-10 accentuates this effect in inactive disease. This change would result in IL-10 increasing the anti-dsDNA antibody production from patients with
inactive disease and decreasing the antibody production from patients with active disease, as was found in this thesis.

In order to test this hypothesis it would be necessary to count the number of plasma cells _ex vivo_, after three days of culture in growth medium and three days growth in IL-10 treated growth media. Plasma cells could be counted using flow cytometry; Syndecan-1 has recently been described as a plasma cell marker (Chilosi _et al._ 1999). However, plasma cells are quite rare in the periphery so this approach may be technically challenging.
Appendices

Publication from this work


Materials

Fluorescein conjugated monoclonal mouse anti-human monocyte CD14, TÜK4 clone, Dako, High Wycombe, UK

Fluorescein conjugated monoclonal mouse IgG2a anti-Aspergillus niger glucose oxidase (negative control), DAK-G05 clone, Dako, High Wycombe, UK

Fluorescein conjugated F(ab')2 fragment of goat anti-mouse immunoglobulins, Dako, High Wycombe, UK

Mouse monoclonal anti-human IL-10 antibody, 23738.11 clone, Research and Diagnostics, Abingdon, UK

Mouse IgG1, anti-MOPC-21 clone, isotype control, Sigma Chemical Company, St. Louis, MO, USA

Mouse IgG1 anti-human interleukin-10 receptor, R-phycoerythrin conjugated, clone 37607.11, Research and Diagnostics, Abingdon, UK

Anti CD14, Murine IgG2a, clone M5E2, Pharmingen, Beckton-Dickenson, USA

Anti CD3, Murine IgG1, clone UCHT1, Pharmingen, Beckton-Dickenson, USA

Fluorescinated annexin V kit, Research and Diagnostics, Abingdon, UK

Anti CD3 R-phycoerythrin conjugated, Murine IgG1, clone UCHT1, Pharmingen, Beckton-Dickenson, USA

Goat anti-human IgG Fab2, Sigma Chemical Company, St. Louis, MO, USA
Goat anti-human IgG alkaline phosphotase conjugated, Sigma Chemicals, St. Louis, MO, USA

Pan anti-mouse IgG coated magnetic beads, Dynal, Oslo, Denmark.

Bovine Serum Albumin, Sigma Chemicals, St. Louis, MO, USA

Casein Sigma Chemicals, St. Louis, MO, USA

**Cytokines**

Human recombinant interleukin 10, Research and Diagnostics, Abingdon, UK

Human recombinant interleukin-10, Immunokontact, Human recombinant interleukin-10, Sigma Chemicals, St. Louis, MO, USA

Human recombinant interleukin-12, Sigma Chemicals, St. Louis, MO, USA

Human recombinant interleukin-12, Research and Diagnostics, Abingdon, UK

Deoxyribonuclease I, Sigma, St. Louis, Missouri, USA

Double stranded DNA from calf thymus, Sigma Chemical Company, St. Louis, MO, USA

EDTA, Sigma Chemical Company, St. Louis, Missouri, USA

Ethanol, BDH Laboratories Supplies, Poole, UK

Fetal calf serum, Sigma Cell Culture, St. Louis, Missouri, USA

Fifty ml tubes from Falcon, Becton Dickenson, USA

Gentamicin, GibcoBRL, Paisley, Scotland

Glutamine, GibcoBRL, Paisley, Scotland

Histopaque®-1077, Sigma Diagnostics, St. Louis, MO, USA

Ninety-six well Maxisorb plates, NUNC, Denmark.

Non-essential amino acids, GibcoBRL, Paisley, Scotland

One ml screw top container from Eppendorf, Hamburg, Germany
Paraformaldehyde, BDH Laboratories Supplies, Poole, UK

Penicillin, GibcoBRL, Paisley, Scotland

P-nitrophenol phosphatase substrate tablets, Sigma Chemicals, St. Louis, MO, USA

Quantitated normal human serum, Pru Procurements, UK

Round bottomed 96-well tissue culture plates, Falcon, Becton Dickenson, USA

Six well plates, polystyrene, Becton Dickinson Labware, Bedford, MA

Sodium Azide, Sigma Chemical Company, St. Louis, MO, USA

Sodium Pyruvate, GibcoBRL, Paisley, Scotland

Streptomycin, GibcoBRL, Paisley, Scotland

Syringe filters (Acrodisc®), 0.2 µm, Gelman Sciences, Ann Arbor, MI
1. GENERAL NON-SPECIFIC MANIFESTATIONS (Gen)

1. Pyrexia
2. Weight loss - unintentional weight loss > 5% in 1 month
3. Lymphadenopathy
4. Fatigue/malaise/weakness
5. Anorexia/nausea/vomiting

CATEGORY A
Pyrexia
plus 2 other

CATEGORY B
Pyrexia
or two other

CATEGORY C
Any other one criterion

CATEGORY D
Previous involvement

CATEGORY E
No involvement

2. MUCOCUTANEOUS DISEASE (Muc)

CATEGORY A - any one of
1. Severe maculopapular, discoid or
   bullous eruption; ie active facial
   and/or extensive (>2/9), scarring
   or causing disability.
2. Angio-oedema
3. Extensive mucosal ulceration

CATEGORY B - any one of
1. Malar erythema
2. Mild maculopapular
   eruption
3. Panniculitis
4. Localised active discoid
   lesions incl. lupus profundus
5. Severe active alopecia
6. Subcutaneous nodules
7. Perniotic skin lesions

CATEGORY C - any one of
1. Peri-ungual erythema
2. Swollen fingers
3. Sclerodactyly
4. Calciosis
5. Telangiectasia
6. Mild alopecia
7. Small mucosal ulceration

CATEGORY D
Previous involvement

CATEGORY E
No involvement
3. CNS DISEASE ATTRIBUTABLE TO LUPUS (Cns)

FIRST ASSESSMENT.

CATEGORY A
Acute, progressive or recurring:
Any one of
1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand mal seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

CATEGORY B
Any one of
1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies

CATEGORY C
1. Episodic migrainous headaches

CATEGORY D
Previous CNS disease

CATEGORY E
No previous CNS disease
CNS DISEASE - SUBSEQUENT ASSESSMENTS

CATEGORY A
Acute, progressive or recurring (scored "worse" or "new").
Any one of
1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand mal seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

CATEGORY B
Any one of the following "new" or "worse" in the last month
1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies
or
Any one of the following "same" or "improving" in the last month on
5. Impaired level of consciousness
6. Psychosis, delirium or confusional state
7. Grand mal seizure

CATEGORY C
1. Episodic migrainous headaches
or
"A" 4-10 or "B" 1-4 "same" or "improving" over the last month.

CATEGORY D
Previous CNS disease

CATEGORY E
No previous CNS disease
4. MUSCULOSKELETAL DISEASE (MsK)

**CATEGORY A**
One or more of
1. Definite myositis (Bohan and Peter)
2. Severe polyarthritis with loss of function (not responsive to steroids <10mg/day, antimalarials, NSAIDS)

**CATEGORY B**
One or more of
1. Arthritis (definite synovitis)
2. Tendonitis

**CATEGORY C**
1. Arthralgia
2. Myalgia
3. Tendon contractures and fixed deformity
4. Aseptic necrosis
5. Mild chronic myositis

**CATEGORY D**
Previous involvement

**CATEGORY E**
No previous involvement

5. CARDIOVASCULAR/ RESPIRATORY DISEASE (Car)

**CATEGORY A**
Cardiac failure or symptomatic effusion plus two other criteria or four from:
1. Pleuropericardial pain
2. Dyspnoea
3. Friction rub
4. Progressive xcr changes - lung fields
5. Progressive xcr changes - heart size
6. ECG evidence of pericarditis or myocarditis
7. Cardiac arrhythmias including tachycardia - >100 in absence of fever
8. Deteriorating lung function: <20% of expected or >20% fall
9. Cytohistological evidence of inflammatory lung disease

**CATEGORY B**
Any two criteria listed under A

**CATEGORY C**
1. Mild intermittent chest pain or one other criterion

**CATEGORY D**
Previous involvement

**CATEGORY E**
No previous involvement
6. **VASCULITIS**

Any one of the following

**CATEGORY A**
1. Major cutaneous vasculitis (including ulcers) accompanied by infarction occurring in previous month
2. Major abdominal crisis due to vasculitis
3. Recurrent thromboembolism (excluding strokes)

**CATEGORY B**
1. Minor cutaneous vasculitis (nailfold vasculitis, digital vasculitis, purpura, urticaria)
2. Superficial phlebitis
3. Thromboembolism (excluding strokes) - first episode

**CATEGORY C**
1. Raynaud's phenomenon
2. Livedo reticularis

**CATEGORY D**
Previous involvement

**CATEGORY E**
No involvement

7. **RENAL DISEASE**

**FIRST ASSESSMENT**

**CATEGORY A**
Two or more of the following provided that 1, 4 or 5 are included
1. Proteinuria (defined as) >1g/24hr or 3+ or 4+ dipstick
2. Accelerated hypertension
3. Creatinine clearance <50ml/min
4. Active urinary sediment (on an uncentrifuged specimen): pyuria (>5wc/hpf); haematuria (>5rbc/hpf) or red cell casts in the absence of infection
5. Histological evidence of active nephritis within the last 3 months (or since the previous assessment if seen less than 3 months ago)

**CATEGORY B**
One of the following
1. One of the category A criteria
2. Urinary dipstick 2+ or more
3. 24hr urinary protein >0.5g but <1g

**CATEGORY C**
One of the following
1. Urinary dipstick +
3. Blood pressure >140/90 (5th phase)
4. Creatinine > 130mmol/l
CATEGORY D
Previous renal involvement

CATEGORY E
No previous renal involvement

RENAL DISEASE - SUBSEQUENT ASSESSMENTS

CATEGORY A
Two or more of the following providing 1, 4 or 5 are included
1. Proteinuria (defined as)
   (a) urinary dipstick increased by 2 or more levels
   or (b) 24 hour urinary protein rising from >0.20g to >1g
   or (c) 24 hour urinary protein rising from >1g by 100% or more
   or (d) newly documented proteinuria of >1g
2. Accelerated hypertension
3. Deteriorating renal function (defined as)
   (a) plasma creatinine >130uM/L and having risen to >130% of
   previous value
   or (b) creatinine clearance having fallen to <67% of previous value
   or (c) creatinine clearance <50ml/min, and last time was >50ml/min
   or was not measured
4. Active urinary sediment (as defined above)
5. Histological evidence of active nephritis (as defined above)

CATEGORY B
One of the following
1. One of the category A criteria
2. Moderate proteinuria (defined as)
   (a) urinary dipstick of 2+ or more
   or (b) 24 hr urinary protein rising from >1g by >50% but <100%
3. Moderate decline in renal function (defined as)
   (a) plasma creatinine >130uM/L or having risen to 115% of
   previous value

CATEGORY C
One of the following
1. 24hr urinary protein >0.25g
2. Urinary dipstick 1+ or more
3. Rising blood pressure (defined as)
   (a) systolic rise of > = 30mm
   (b) diastolic rise of > = 15mm
   (providing the recorded values are > 140/90)

CATEGORY D
Previous renal disease

CATEGORY E
No previous renal disease
8. **HAEMATOLOGICAL DISEASE (Hae)**

**CATEGORY A**  
One of the following  
1. wcc < 1000  
2. platelet count < 25  
3. haemoglobin < 8

**CATEGORY B**  
One of the following  
1. wcc < 2500  
2. platelet count < 100  
3. haemoglobin < 11  
4. evidence of active haemolysis  
   (raised bilirubin +/− reticulocyte count and positive Coomb's test)

**CATEGORY C**  
One of the following  
1. wcc < 4000  
2. lymphocyte count < 1500  
3. platelet count < 150  
4. Coomb's test positive but no evidence of active haemolysis  
5. Evidence of circulating lupus anti-coagulant detected by functional assays

**CATEGORY D**  
Previous involvement

**CATEGORY E**  
No involvement
The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus

E.M. HAY, P.A. BACON, C. GORDON, D.A. ISENBERG, P. MADDISON, M.L. SNAITH, D.P.M. SYMMONS, N. VINTER and A. ZOMA

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Summary

The British Isles Lupus Assessment Group (BILAG) index is a computerized index for measuring clinical disease activity in systemic lupus erythematosus (SLE), which was developed according to the principle of the physician's 'intention to treat'. The index allocates separate alphabetic scores to each of eight organ-based systems; a total score is not calculated. This study demonstrated good between-rater reliability for the BILAG index for each organ-based system. There was no evidence of bias between observers. The BILAG index had good overall sensitivity (87%) and specificity (99%) when compared with the 'gold standard' criterion (starting or increasing disease-modifying therapy). There were high positive predictive values overall (80%), and for each organ-based system, with the exception of the neurological system.

Introduction

SLE is a complex multi-system disease; this complexity makes the disease difficult to monitor. In particular, there are problems in quantifying disease activity across systems, and in differentiating potentially reversible organ dysfunction (due to active disease) from irreversible organ damage. Defining the term 'activity' is also a problem. Poor correlations were found between physicians' scores when they assessed patients using a semi-quantitative clinical rating scale. In order to enable physicians to communicate using a common language, standardized quantitative measures of SLE clinical disease activity are required. Such scales would be of value in clinical trials, in longitudinal studies of outcome, or the evaluation of immunological markers.

More than 60 scales for measuring clinical disease activity in SLE have been developed and used in a variety of studies. Only recently, however, have scales have been tested for reliability and validity. A scale should give the same result when two or more physicians assess the same patient (between-rater reliability), and when one physician assesses a patient with a steady clinical state at different points in time (within-rater reliability). Variability may result from either systematic (bias) or random measurement errors. Within-rater reliability is difficult to test because raters may remember the previous score if the time between assessments is short or, if the assessments are a long time apart, the patient's clinical condition may have changed. However, because within-rater reliability contributes to between-rater reliability, if between-rater reliability is good then within-rater reliability can be also assumed to be good.

The validity of clinical activity scales can be assessed in several ways. First, a scale should appear to measure disease activity, and the individual variables should be measured in a generally accepted way (face validity). Second, a scale should include an adequate number of variables.
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