ABSTRACT

Reactive oxygen species have been implicated in the induction of autoimmunity via the initiation of protein oxidation reactions leading to the recognition of antigen as non 'self'. In systemic lupus erythematosus, the source of reactive oxygen species capable of inducing damage to antigens may be cellular but could also involve those generated by ultraviolet in sunlight. A key feature of systemic lupus erythematosus is the development of photosensitive skin lesions and these have been positively associated with the presence of Ro60 autoantibodies. The autoantibody response to Ro60 in systemic lupus erythematosus sera has been characterised and is a high affinity antibody of predominantly IgG1 subclass. This implicates a T cell dependent process involving antigen presentation to T helper cells via the MHC class II antigen-presenting pathway. To date, there has been no work reported on T cell responses to Ro60 or systemic lupus erythematosus autoantigens modified by reactive oxygen species in systemic lupus erythematosus subjects.

The primary aim of this project was to investigate if T cells isolated from systemic lupus erythematosus subjects were able to respond to purified human recombinant Ro60 after it has been modified by reactive oxygen species. In addition to cell activation, cell death also plays an important role in antigen activated immune responses and dysfunctions in cell death regulation have been implicated in systemic lupus erythematosus. Therefore in addition to antigen induced cell activation, cell death was also investigated. Initial work focused on purification of soluble recombinant Ro60 after overexpression in E. coli. Ro60 was then exposed to reactive oxygen species via ultraviolet B irradiation and metal catalyzed oxidation and immunochemical techniques were applied to detect aggregation, fragmentation and carbonyl formation as markers of protein oxidation. None of these markers were induced by ultraviolet B irradiation but metal catalyzed oxidation induced carbonyl formation on a high molecular weight aggregate of Ro60. Free radical scavengers and metal ion chelators suggested the mechanism of oxidation to be a ‘caged reaction’ as opposed to a reaction involving free hydroxyl radical formation.

A method was established to measure T cell activation analysing viable interferon gamma secreting and interleukin-4 secreting leukocytes by flow cytometry using a non-specific T cell activator as a positive control. This was used to investigate T cell activation of peripheral blood mononuclear cells obtained from systemic lupus erythematosus subjects after stimulation in vitro with Ro60 that had been exposed to reactive oxygen species. In addition cells undergoing apoptotic and necrotic cell death were also detected by flow cytometry after staining with annexin V and propidium iodide. The data were compared to that from age- and sex-matched healthy controls. There was no significant difference increase or decrease in T cell activation or the level of cell death between the two groups after stimulation with reactive oxygen species modified Ro60. The data represent the highly individual response of subject’s immune cells to antigen stimulation. The reactive oxygen species modified Ro60 used in these investigations represented only a model system to test the hypothesis of reactive oxygen species modification to autoantigen as an initiating factor in autoimmunity.

The methodology established may be applied to other autoantigens in systemic lupus erythematosus or other autoimmune conditions to test a whole spectrum of reactive oxygen species modifications to proteins in addition to other posttranslational modifications which may be involved in the initiation of autoantigen as non 'self'.

II
To my parents
ACKNOWLEDGEMENTS

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The effect of metal catalyzed oxidation on carbonyl formation detected by Western blotting on proteins present within NDF cell lysates

Possible mechanism of metal ion-catalyzed oxidation of protein

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PBMC isolated from a healthy volunteer was used to detect IFNγ+ (CD4- and CD4+) lymphocytes after stimulation with SEB for 6 hrs by flow cytometry.

PBMC isolated from a healthy volunteer was used to detect IFNγ+ and or IL-4 lymphocytes after stimulation with SEB for 6 hrs by flow cytometry

IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes isolated from two Ro positive subjects after antigen stimulation with PMA/I (R1) and SEB (R2) for 16 hours.

IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes isolated from three Ro positive subjects (R3, R4 and R5) and age and sex-matched controls (C1, C2 and C3) after antigen stimulation with SEB and Ro60 10μg/ml for 16 hours.

IFNγ+ and IL-4+ secreting lymphocytes isolated from a Ro positive subject (R6) after antigen stimulation with SEB 4 hours.

IFNγ, IL-4 and IFNγ & IL-4 secreting lymphocytes isolated from a Ro positive subject (R7) after antigen stimulation with SEB and 3 concentrations of Ro60 (5μg/ml, 10μg/ml 20μg/ml) following 6 and 16 hours incubation were investigated.

IFNγ, IL-4 and IFNγ & IL-4 secreting lymphocytes isolated from three Ro positive subjects (R7, R8 and R9) and age and sex-matched controls (C5, C6 and C7) after antigen stimulation with SEB and Ro60 (5μg/ml, 10μg/ml 20μg/ml) following 6 hours incubation

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IL-4 secreting lymphocytes isolated from a Ro positive subject (R5) and age and sex-matched controls (C10 and C3) after antigen stimulation with SEB and Ro60 (10μg/ml) following 6 and 16 hours incubation

IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8 and C11) after antigen stimulation with SEB and Ro60 10μg/ml for 6 hrs.

IL-4 secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C10 and C11) after antigen stimulation with SEB and Ro60 10μg/ml for 6 hours

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<td>APS</td>
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<td>bp</td>
<td>base pairs</td>
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<td>bovine serum albumin</td>
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<td>°C</td>
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<td>foetal calf serum</td>
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<td>glomerular basement membrane</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)</td>
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<td>HNE</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>hydroperoxyl</td>
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<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<td>kb</td>
<td>kilobase</td>
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<tr>
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<td>kilodaltons</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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XVIII
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<th>Abbreviation</th>
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<td>LN</td>
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<tr>
<td>MBP</td>
<td>maltose binding protein</td>
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<td>MACS</td>
<td>magnet assisted cell separation</td>
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<td>MCTD</td>
<td>mixed connective tissue disease</td>
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<td>MCO</td>
<td>metal catalyzed oxidation</td>
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<td>MDA</td>
<td>malondialdehyde</td>
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<td>MED</td>
<td>minimal erythemal dose</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>min</td>
<td>minutes</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulfonic acid</td>
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<td>NC1</td>
<td>non-collagenous globular</td>
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<td>NL</td>
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<td>NP-40</td>
<td>Nonidet P-40</td>
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<td>OD</td>
<td>optical density</td>
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<td>superoxide</td>
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<td>OH$^-$</td>
<td>hydroxyl</td>
</tr>
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<td>ONOO$^-$</td>
<td>peroxynitrite</td>
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<td>O$_3$</td>
<td>ozone</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PEG</td>
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<td>pers. comm.</td>
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<td>Pyrococcus furiosus</td>
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<td>propidium iodide</td>
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<td>PMFS</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>PMA/I</td>
<td>phorbol 12-myristate 13-acetate/ionomycin</td>
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<td>PS</td>
<td>phosphodidylyserine</td>
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<td>polyvinylidene difluoride</td>
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<td>Ro ribonuclearprotein</td>
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<td>RNP</td>
<td>ribonuclear protein</td>
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<td>RO$^-$</td>
<td>alkoxy radical</td>
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<td>RO$_2^-$</td>
<td>peroxyl radical</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium 1640</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RT</td>
<td>room temperature</td>
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<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
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<tr>
<td>SCLE</td>
<td>Subcutaneous Lupus Erythematosus</td>
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<tr>
<td>SDS</td>
<td>sodium dodecy1 sulphate</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEB</td>
<td>Staphylococcal Enterotoxin B</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
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<tr>
<td>TBE</td>
<td>tris-borate EDTA</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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XIX
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<th>Abbreviation</th>
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<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TE</td>
<td>tris-EDTA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>UVA</td>
<td>ultraviolet A (320nm-400nm)</td>
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<td>UVB</td>
<td>ultraviolet B (280-320nm)</td>
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<td>UVC</td>
<td>ultraviolet C (100-280nm)</td>
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<td>UP</td>
<td>ultrapure</td>
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CHAPTER 1:

INTRODUCTION
1.0 Introduction

1.1 Systemic lupus erythematosus - an overview

Autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren’s syndrome (SS) are rheumatic diseases characterised by the production of autoantibody. These diseases present with chronic inflammation, may be life-long, are debilitating and patients show increased mortality. SLE is a systemic autoimmune disease where the immune system attacks organs and tissues throughout the body as opposed to organ-specific autoimmune disease where one particular organ is involved. This results in a variety of symptoms making the diagnosis of the disease difficult to define, and for the purpose of identifying patients in clinical studies a set of criteria has been developed for the classification of SLE (Table 1.1). This provides an example of the vast array of symptoms associated with this disease, ranging from development of skin lesions exacerbated by exposure to ultraviolet (UV) light, to more severe manifestations. (for example the development of renal disease).

1.2 Prevalence

A study of the incidence of SLE in the general population was conducted in Birmingham, UK during 1991 and 1992 and the prevalence of 28 per 100,000 was recorded (Johnson et al, 1995). The disease is however more prevalent in particular ethnic groups. For example, in the same study the prevalence in those of Afro-Caribbean origin was 400 per 100,000 compared to 47 in 100,000 in those of Asian origin. SLE is also more common in women than men where the gender distribution is 9:1 (female/male) and is also more prevalent in women during the reproductive years (Ansar et al, 1985). The disease may be exacerbated by the use of oral contraceptive (Beaumont et al, 1989) and often flares during pregnancy (Hayslett et al, 1985) suggesting a hormonal influence on the disease process.

1.3 Autoantibodies

In SLE many autoantibodies to antigens are present and only a few more common examples are described here. Autoantigens are of diverse cellular location and those to extracellular antigens have also been detected. The types of autoantibodies observed are often related to particular manifestations of the disease. The initial identification of autoantibody in SLE came from observations of the interaction of cell nuclei with globulin from SLE serum (Friou et al, 1957). These antibodies were found to interact with several components of the nucleus including antibodies...
<table>
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<tr>
<th>Criterion</th>
<th>Definition</th>
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<tr>
<td>1. Malar rash</td>
<td>Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds</td>
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<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions</td>
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<td>3. Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation</td>
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<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration, usually painless, observed by physician</td>
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<tr>
<td>5. Arthritis</td>
<td>Nonerosive arthritis involving 2 or more peripheral joints, characterised by tenderness, swelling or effusion</td>
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</table>
| 6. Serositis       | a) Pleuritis- convincing history or pleuritic pain or rub heard by a physician or evidence of pleural effusion; or  
|                    | b) Pericarditis- documented by ECG or rub or evidence of pericardial effusion                                                              |
| 7. Renal disorder  | a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed; or  
|                    | b) Cellular casts – may be red cell, haemoglobin, granular, tubular, or mixed                                                               |
| 8. Neurologic disorder | a) Seizures- in the absence of offending drugs or known metabolic derangements, eg uremia, ketoacidosis, or electrolyte imbalance; or  
|                    | b) Psychosis- in the absence of offending drugs or known metabolic derangements, eg uremia, ketoacidosis, or electrolyte imbalance      |
| 9. Hematologic disorder | a) Hemolytic anemia- with reticulocytosis; or  
|                    | b) Leukopenia- less than 4,000/mm³ total on 2 or more occasions; or  
|                    | c) Lymphopenia- less than 1,500/mm³ total on 2 or more occasions; or  
|                    | d) Thrombocytopenia- less than 100,000/mm³ in the absence of offending drugs                                                             |
| 10. Immunologic disorder | a) Positive LE cell preparation; or  
|                    | b) Anti-DNA: antibody to native DNA in abnormal titre; or  
|                    | c) Anti-Sm: presence of antibody to Sm nuclear antigen; or  
|                    | d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test |
| 11. Antinuclear antibody | An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with 'drug-induced lupus' syndrome |

Table 1.1: The 1982 revised criteria for the classification of systemic lupus erythematosus, adapted from Tan et al, 1982. SLE results in a variety of symptoms (as shown in the table) making the diagnosis of the disease difficult to define. Therefore the original criteria for the classification were developed for the purpose of identifying patients with SLE for clinical studies (a revised version is shown here). Using these criteria a person is classified as having SLE if they present with any 4 or more of the 11 criteria, serially or simultaneously, during any interval of observation.
to a major antigen recognized as a histone DNA complex (Holborow et al, 1957), and also to so-called extractable nuclear antigens to ribonuclear proteins (RNP) consisting of small nuclear RNA species and associated proteins (Maddison and Reichlin, 1977). The anti-nuclear antibodies (ANA) test is often used as an indication of whether a subject has SLE as nearly all SLE subjects have been found to have ANA but it is not conclusive as this test may be positive for other rheumatic autoimmune conditions such as progressive systemic sclerosis and polymyositis (reviewed by Reichlin and Harley, 1997). This test superseded the original test used for the identification of SLE, the LE cell, first described by Hargreaves et al, 1948 where blood polymorphonuclear cells were seen to ingest large quantities of nuclear material from other white blood cells, now suggested to represent that damaged by anti-nuclear antibodies.

The most characteristic autoantibody in SLE is the presence of autoantibodies to dsDNA, these antibodies have been shown to present in 50 out of 51 cases of SLE and only one from 660 control sera from another autoimmune condition (Ceppellini et al, 1957; Swaak et al 1979). Further studies showed that active lupus nephritis was associated with high levels of anti-dsDNA (Ter Borg et al, 1990). The autoantibodies in SLE are not confined to nuclear components; autoantibodies are also targeted to cell membrane antigens, for example, surface molecules of lymphocytes (Butler et al, 1972) which have been associated with lymphopenia in SLE (Winfield et al, 1975); and also to serum components, for example, antibodies to the Fc portion of immunoglobulins (Singer, 1961). A common autoantibody in SLE, which exists mainly in the cytoplasm, is the ribonuclear protein, Ro (Table 2). Due to its cytoplasmic location it is sometimes seen in patients whose conventional antinuclear tests are negative (Maddison et al, 1981). This protein autoantibody has been shown to be associated with photosensitivity in SLE (Mond et al, 1989). This protein will be used as a model SLE protein autoantigen in this thesis and therefore the following section discusses this autoantigen in more detail.

1.4 The Ro/SS-A and La/SS-B Autoantigens

These autoantigens were identified by Reichlin, et al in 1969 following the characterisation of a soluble cytoplasmic antigen reactive with sera from patients with SLE. The names of the antigens, Ro and La, were taken from the names of the first patients in which they were identified. In 1975 two antigens, SS-A and SS-B, were similarly identified using sera from patients with Sjörgen's syndrome by Alspaugh and Tan, 1979. It was subsequently discovered that Ro and SS-A, and La and SS-B, were identical antigens after experiments involving the exchange of sera between the two groups (Alspaugh and Maddison, 1979). In autoimmune disease these autoantibodies are most
frequently found in patients with Sjögren’s syndrome and with particular variants of SLE (Harley, et al. 1986). These include subacute cutaneous lupus erythematosus (SCLE), which is diagnosed by the presence of a photosensitive rash, where clinical association has been linked to the presence of anti-Ro antibody (Sontheimer et al., 1982) and neonatal Lupus (NL). In NL the autoantibodies are transferred from the mother to the unborn child across the placenta. This has been associated with two clinical syndromes in the neonate, complete congenital heart block (fortunately very rare and usually not life threatening) and skin lesion similar to those found in adult SCLE, which subside once the maternal antibody has diminished (Tseng and Buyon, 1997). The frequency of these autoantibodies in various autoimmune diseases and those described is shown in Table 1.2. Generally these autoantibodies present together; however there have been cases of anti-Ro antibodies observed in the absence anti-La (Slobbe et al., 1991).

<table>
<thead>
<tr>
<th></th>
<th>Anti-Ro/SS-A</th>
<th>Anti-La/SS-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sjögren’s syndrome</td>
<td>85-95</td>
<td>68-87</td>
</tr>
<tr>
<td>SLE</td>
<td>25-60</td>
<td>19-30</td>
</tr>
<tr>
<td>Subacute cutaneous Lupus</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>Erythematous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Lupus</td>
<td>96</td>
<td>86</td>
</tr>
<tr>
<td>Dermatomyositis/Polymyositis</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Mixed connective tissue disease</td>
<td>5-10</td>
<td>Rare</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>2-10</td>
<td>1</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>18</td>
<td>Rare</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1.2: Frequency (%) of anti-Ro/SS-A and anti-La/SS-B autoantibodies in various autoimmune diseases; adapted from Hiepe and Dorner, 1999. These are common autoantibodies in SLE and also with particular variants of SLE including subacute cutaneous lupus erythematosus and neonatal lupus but are most frequently found in patients with Sjögren’s syndrome. They are also found (although less frequently) in other autoimmune rheumatic diseases such as dermatomyositis/polymyositis, mixed connective tissue disease, rheumatoid arthritis and primary biliary cirrhosis but are rarely found in healthy subjects. In most cases both autoantibodies are found, however there cases of anti-Ro antibodies observed in the absence anti-La.
1.5 The Ro RNP complex

The human autoantigens Ro and La were found to be associated with one of four small cytoplasmic Y RNA molecules (hY1 - hY5 RNA; hY2 RNA is a degradation product of hY1) transcribed by RNA polymerase III forming a Ro RNP complex (Hendrick et al, 1981; and Wolin et al, 1984). The secondary structure of hY RNAs were predicted from their sequence to be folded into a long stem formed by base-pairing of the 5' and 3' ends. The oligo(U) stretch at their 3' end which is usually lost upon RNA maturation was shown to be retained in these molecules. A highly conserved bulged helix in the stem was shown to be protected from RNase degradation possibly by the presence of protein (Kato et al, 1982; and Wolin et al, 1984). Further research in to the properties of these autoantigens showed the Ro autoantigen particle to be composed of two components; a 60-kDa protein Ro60 and 52-kDa protein Ro52 (Ben Cherit et al, 1988) and that binding of the La protein to RNA polymerases III transcripts was shown to be necessary for correct termination of transcription. (Gottlieb et al, 1989). The analysis of protein-RNA interaction within RoRNP complexes was studied by Pruijn, et al in 1991 and the binding regions of Ro60 and La proteins were mapped to the highly conserved bulged helix in the stem and the oligo(U) stretch at the 3' end of the Y RNA molecules respectively. Further work suggested that the association of Ro52 with the RoRNP complex was mediated by Ro60 and a model structure of the RoRNP complex was proposed. The larger than expected size of the RoRNP complex of between 200 and 350 kDa (Boire and Craft, 1990; and Kelekar et al, 1994) and findings of cDNAs encoding alternatively spliced variants of Ro and La (Chan et al, 1994 and Hilker et al, 1995) lead to further study of the fine detail of the RoRNP complex using biochemical purification and in vitro reconstitution techniques. Several other proteins that appear to bind Y RNA were identified (Fabini et al, 2000) and the current hypothetical model for human Y RNAs based on these observations is shown in Figure 1.1 (Fabini et al, 2000).

Despite the major RoRNP components Y RNA, Ro60 and La being conserved during evolution (indicating an important role in the cells), the function of the RoRNP complex has yet to be assigned. Comparison of Y RNA sequences which have been found in cells from several vertebrate species (Farris et al, 1995) and in C. Elegans (van Horn et al, 1995) have shown this molecule to be highly conserved during evolution. The La protein has also been well conserved during evolution, as demonstrated by sequence comparison of La encoding cDNAs in several species including D. melanogaster and S. cerevisiae (Yoo et al, 1994). Two sequences of the human Ro60 protein have been obtained, these are essentially identical apart from a small region at the amino terminus (Deutscher et al, 1988; and Ben-Chetrit et al, 1989) and show some homology with the polypeptides
Figure 1.1: Hypothetical model for human Y1 RNP adapted from Fabini et al, 2001. Ro and La autoantigens are associated with a Y RNA molecule transcribed by RNA polymerase III forming a Ro RNP complex. The secondary structure of Y RNA is predicted be folded into a long stem formed by base-pairing of the 5‘ and 3’ ends. The binding regions of 60-kDa Ro60 and 52-kDa Ro52 protein autoantigens have been mapped to the highly conserved bulged helix in the stem. The oligo(U) stretch at the 3’ end of the Y RNA molecule (usually lost upon RNA maturation and shown to be retained in these molecules) has been shown to be the binding region for the 42-kDa La protein autoantigen. Further study of the RoRNP complex have suggested several other proteins that appear to bind the Y RNA in the region of the second loop in the Y RNA stem as shown in the diagram which represents the current hypothetical model for human Y RNAs.

encoded from Ro60 cDNAs found in X.laevis (O’Brien et al, 1993) and C.elegans (van Horn et al, 1995). The role of the RoRNP complex in cells is not known, however there have been several reports that suggest two potential roles for La. Firstly, in the nucleus as a regulatory transcription factor protein (Gottlieb et al, 1989; and Maria, 1996) and possibly related to this function, La has been shown to be able to unwind DNA-RNA hybrids and dsRNA in an ATP-dependent manner (Bachman et al, 1990; and Huhn et al, 1997) A second cytoplasmic role in the regulation of translation has been indicated by the involvement of La in the translation of certain viral RNAs (Meerovitch et al, 1993; and Svitkin, et al, 1994) and more recently La was reported to be associated with small ribosomal subunits (Peek, et al, 1996). Less is known about the function of the other components of the RoRNP complex. The Ro60 protein has been suggested to function in a quality control or discard pathway for 5S rRNA biosynthesis where it has been found in association with variant 5S rRNA precursors in X. laevis oocytes (O’Brien and Wolin, 1994; and Shi et al,
In addition to the association with Y RNA in the cytoplasmic RoRNP complex, La has been shown to reside (consistent with the suggested role in transcription and also translation) in the nucleus (Smith et al, 1985) and in the cytoplasm associated with small ribosomal subunits (Peek, et al, 1996).

1.6 Treatment of SLE

Treatment is currently limited to non-specific anti-inflammatory or immunosuppressive therapy and an understanding of the mechanisms involved in the development of SLE would enable a more targeted therapeutic approach. For controlling mild SLE symptoms, non-steroidal anti-inflammatory drugs for example Aspirin and Ibuprofen (Karsh et al, 1980) which work by inhibiting chemicals, prostaglandins and thromboxanes associated with inflammation (Samuelsson, 1987) are prescribed. For reducing chronic inflammation, corticosteroids (for example prednisolone) are often administered (Hench et al, 1949); these have powerful anti-inflammatory and immunosuppressive effects decreasing the activity of white blood cells, production and action of cytokines, and the amount of complement proteins in the blood (Parillo and Fauci, 1979). For the more serious symptoms such as lupus nephritis, immunosuppressive therapy by the use of cytotoxic agents is often used for example cyclophosphamide and azathioprine (Felson and Anderson, 1984). These chemicals work by direct effects on immune cells blocking the early stages of the immune response (Fox and McCune, 1994). The anti-malarial drug hydroxychloroquine is also commonly used for the treatment of SLE (Combleet, 1956). The reason why anti-malarials are effective in treating SLE is still largely unknown, despite one of the first descriptions of their use as a treatment in SLE being reported more than 100 years ago (Payne, 1894).

The classical drugs for the treatment of SLE are effective at controlling the disease symptoms. However, there are risks associated with the side effects of these drugs, particularly those associated with cytotoxic properties. Future treatment using drugs which specifically target immune abnormalities in SLE are being researched. For example an anti-CD40L antibody has been shown to be effective at reducing anti-DNA antibodies in lupus prone mice by blocking CD40:CD40L interactions between B cells and T helper cells (Early et al, 1996). Also recombinant DNase has been used in a trial to as a potential therapy for LN, and it has been proposed that it might prevent the deposition of anti-DNA immune complexes. However, the treatment was tolerated by the immune system but did not reduce the number of circulating DNA complexes (Davis et al, 1999). Finally, a treatment using a B cell toleragen developed by La Jolla Pharmaceuticals called LJP394 designed to reduce anti-dsDNA production has been investigated and demonstrated a decrease in
anti-DNA levels shortly after infusion and its potential use is still being evaluated (Weisman et al, 1997).

1.7 Pathogenesis
1.7.1 Genes and Environment
Both genetic and environmental influences have been suggested to play a role in the development of SLE. This can be demonstrated by the estimate of twin concordance in SLE which is estimated to be 24% between monozygotic twins, compared to only 2% between dizygotic twins (Deapen et al, 1992). The fact that there is not complete concordance between monozygotic twins suggests the involvement of environmental factors as well as genes in the disease. Among the genes already known to be involved in the predisposition of SLE are genes linked to the HLA-DR and DQ loci, containing genes which code for MHC class II molecules. One of the first associations to be reported was an increase in the frequency of HLA-DR2 and DR3 in Caucasian SLE patients (Reinerten et al, 1978). Also genes encoding components of the complement system for example, the C4A null allele is more frequent in SLE and has been associated with complement abnormalities (Batchelor et al, 1987).

A classical example for the role of a particular gene in development of lupus is the MRL strain of mice which spontaneously develop SLE. These mice were found to have a mutation in the lpr gene resulting in incorrect expression of Fas protein and deficiency of apoptosis (Takahashi et al, 1994). Restoration of Fas expression in transgenic mice was shown to ameliorate the disease (Wu et al, 1994). To date no evidence has been found for Fas mutations in humans with SLE. However, a mutation in Fas that is associated with lymphoproliferative syndrome has been found in humans where the clinical symptoms are very similar to those in the lpr mouse (Fisher et al, 1995).

Environmental influences are also thought to play a role in the development of SLE. The observation that a number of drugs cause a lupus-like disease have led to research into chemical and environmental triggers as causes of lupus. Since the initial observation in a subject who developed lupus-like symptoms after he had been treated with sulphadiazine (Gold, 1951) several drugs have been identified which cause a similar response and the condition has been termed drug-induced lupus (Fitzler, 1994). Other examples of environmental influences include dietary factors. In Cynomolgus macaques fed alfalfa sprouts an SLE like illness developed where the amino acid L-canavanine was shown to play a role (Malinow et al, 1982). Viral infections have also been
implicated in the induction of SLE and other autoimmune disease of particular note is the association found between SLE and Epstein Barr virus (James et al, 1997).

1.7.2 Autoantibodies

Whether autoantibodies which occur in SLE and other autoimmune diseases are directly involved in the induction of disease or are produced as a consequence of the disease process has been a central area of research into the pathogenesis of SLE. Several studies have implicated autoantibodies as playing an active role in the development of the disease. Anti-DNA antibodies in particular have been studied due to their association with LN (Swaak et al, 1979). The levels of these autoantibodies have been shown to be increased or decreased in relation to nephrotic exacerbations and remissions of the disease and raising anti-DNA levels have been used as an indicator of upcoming exacerbation so that treatment could be administered to prevent such flares (Bootsma et al, 1991). It has been also shown that the presence of anti-DNA antibodies can be first detected in the circulation up to 5 years prior to the development of SLE (Swaak et al, 1985). Further evidence of implication of anti-DNA in the propagation of renal damage is elution of antibodies to DNA from the effected kidneys (Sugisaki and Takase, 1991), and anti-DNA has been shown to bind to the glomerular basement membrane when perfused into rat kidneys (Termaat et al, 1992).

Anti-Ro has also been implicated in the induction of disease in neonatal lupus syndrome where CHB occurs in 5% of anti-Ro positive mothers. In this condition, anti-Ro is passed to the foetal circulation via the placenta and results in neonatal lupus syndrome (Tseng et al, 1997). Maternal anti-Ro has been eluted from the affected heart tissue where it reacts with autoantigen on the surface of the affected foetal heart (Horsfall et al, 1991), implicating anti-Ro in the development of CHB.

These examples show that autoantibodies have the potential to be involved in the pathogenesis of disease but for the majority of autoantibodies evidence has not been found and this suggests that autoantibody production in SLE might be a consequence of another underlying immune dysfunction.

1.7.3 Autoantigens in SLE

Whether autoantibody production on SLE is the cause or consequence of the disease process is an important question in understanding the disease pathology. The characteristics of the immune response in SLE suggest that it is driven by the autoantigen itself. However the initiating antigens that drive the development of the disease are yet to be identified and this represents a further area of SLE research. The characteristics of an antigen driven response have been observed in the cellular
responses to autoantigens from SLE subjects. These included activation of parallel B and T cell responses to the same antigen (Holyst et al, 1997), epitope spreading in which the response to antigen becomes more diverse as the response persists (Arbuckle et al, 1999) and increased affinity of antibodies produced during an immune response to antigen (Hirose et al, 1993). A mechanism for how normal cellular antigens could become exposed to the immune system and therefore be available to initiate autoimmune response if tolerance was some how bypassed was suggested by Casciola-Rosen et al, 1994. They observed that autoantigens targeted in SLE were clustered on two populations of surface blebs on keratinocytes dying from apoptosis. Since these observations the role of apoptosis in the pathogenesis of SLE has lead to several hypotheses.

1.7.4 Apoptosis - an overview

The cells of the body are constantly being exposed to chemical and physical injury. Irreversible cell injury results in cell death (necrosis) and leaves behind extensive cellular debris that when removed results in the activation of an inflammatory response. However a whole new field of research emerged following the discovery of a different mode of cell death in which the cell activates an internal death programme (Kerr et al, 1972). A characteristic of this form of cell death is the break-up of the entire cell into apoptotic bodies without the release of cell contents. The apoptotic bodies are removed by phagocytic cells without the activation of an inflammatory response (Fadock et al, 1993). A basic function of this complex form of cell death is deletion of unwanted cells, for example during embryonic development and also those in normal tissue (Hakem et al, 1998). The mechanisms of apoptosis have been extensively studied and three major apoptosis-inducing signals have been identified. These are extracellular stimuli converging at cell membrane receptor proteins (Scaffidi et al, 1998), intracellular stimuli converging at the mitochondria leading to the release of cytochrome C (Li et al, 1997) and extrinsic cytotoxic granules delivering enzyme activators to target cells (Andrade et al, 1996). All these signals result in the activation of a caspase proteolytic cascade which orchestrates the execution of apoptotic cell death (Martin and Green, 1995). The process has been shown to be tightly controlled by the identification of proteins shown to be involved in the regulation of the process (Pellegrini and Strasser, 1999).

1.7.5 Apoptosis and the immune system

Apoptosis is essential in maintaining the effective function of the immune system where it plays a major role in the development of tolerance to self antigen. Central tolerance is the main mechanism in the development of tolerance to self antigen, where T cells migrate to the thymus from the bone marrow and interact with peptide-MHC complexes via the T cell receptor. Here, those interactions
with very low affinity (no survival signal) and also of high affinity (death signal) are negatively selected by the process of apoptosis (Surh and Sprent, 1994). The remaining T cells that bind with intermediate affinity are positively selected for maturation and migrate to the periphery; the mechanisms of central tolerance are illustrated in Figure 1.2 A.

Mechanisms of tolerance also exist to prevent activation of self autoreactive T cells which fail to be removed by central tolerance and these are illustrated in Figure 1.2 B. Again, apoptosis is a central mechanism in maintaining tolerance where activated T cells which can express Fas or its cognitive ligand and interaction of these molecules can induce deletion of T cells by apoptosis (Dhein et al, 1995). Other examples of peripheral tolerance include immunological ignorance where the level of antigen may too low to activate T cells (Ferber et al, 1994) or physical separation which exists between antigen and its reactive T cell (Barker and Billingham, 1977). Furthermore the regulation of T cell activation may play a role in peripheral tolerance, for example cytotoxic-T-lymphocyte-associated protein 4 has been shown to be involved in inhibition of the activation of T cells (Luhder et al, 2000).

In addition to a role in tolerance, apoptosis is also involved in regulating the number of cells in the lymphocyte population after antigen activation. Following activation, T cells rapidly proliferate and after reaching maximum numbers begin to decline rapidly, some migrate to the nonlymphoid tissues where T cells remain as effector memory T cells (Masopust et al, 2001) and the rest are rapidly removed by apoptosis by a process involving Fas or TNF-α signalling, known as activation induced cell death (Shi et al, 1989). More recently activated T cell autonomous death was described as a programmed and regulated T cell death process which occurred independently of the Fas- and TNF-α pathway (Badovinac et al, 2002). The process of apoptosis is therefore also shown to be essential in maintaining lymphocyte homeostasis.

1.7.6 Dysfunctional apoptosis in SLE

There have been several studies that have associated dysfunctional apoptosis with the disease SLE, however the relationship is not a straightforward one linking SLE with increased or impaired levels of apoptosis. Indeed apoptosis is a multi-step process and abnormalities may even exist where too much or too little apoptosis could occur simultaneously. One theory is that if apoptotic material exceeds the body’s ability to dispose of it via non-inflammatory processes it may act as an antigen to drive the development of SLE. Studies have shown that apoptotic material can be presented to the immune system to induce an immune response (Ronchetti et al, 1999) and with reference to SLE, it
has been show that mice inoculated with apoptotic material were able to generate autoantibodies associated with those found in SLE (Mevorach et al, 1998).

**Figure 1.2** A Central tolerance mechanisms adapted from Kamradt et al 2001. Apoptosis has a major role in the development of tolerance to self antigen and the main mechanism in the development of tolerance to self antigen is central tolerance. During this process T cells migrate to the thymus from the bone marrow and interact with peptide-MHC complexes via the T cell receptor. Those interactions with very low affinity (no survival signal) and also of high affinity (death signal) are negatively selected by the process of apoptosis and the remaining T cells that bind with intermediate affinity are positively selected for maturation and migrate to the periphery.

It was the work of Cascicola-Rosen et al, 1994 that showed that autoantigens targeted in SLE were clustered on two populations of surface blebs on keratinocytes cells dying from apoptosis which suggested a potential mechanism for exposure of normal cellular material to the immune system and suggested a possible link between defective apoptosis and SLE. In addition the actual process of apoptosis has been suggested to be involved in the development of autoantigens where the majority of autoantigens in systemic autoimmune diseases are cleaved by granzyme B during cytotoxic lymphocyte granule induced death. In contrast, non-autoantigens were found not to be cleaved by granzyme B but were cleaved to produce fragments identical to those generated in other forms of apoptosis (Cascicola-Rosen et al, 1999). Further evidence linking autoantigen generation to apoptosis has been found in SLE where autoantibodies have been shown to target apoptosis-specific epitopes of an SLE autoantigen in some subjects ie, apoptotic U1-70KD is antigenically distinct
from the intact form of the U1-70KD molecule (Greiginger et al, 2000) and immunity to U1-70KD appears to develop before that to other components of the same complex (Greiginger and Hoffman, 2001).

Figure 1.2 B. Peripheral tolerance mechanisms adapted from Kamradt et al 2001. Mechanisms of tolerance also exist in the periphery to to prevent activation of autoreactive T cells which fail to be removed by central tolerance. The level of antigen may too low to activate T cells and here immunological ignorance plays a role, also physical separation which can exist between antigen and its reactive T cell which helps to maintain tolerance in the periphery. T cell deletion may also occur via expression of Fas or its cognitive ligand and interaction of these molecules leading to apoptosis providing a mechanism for peripheral tolerance by deletion of autoreactive T cells. Finally inhibition of the activation of T cells may occur cytotoxic-T-lymphocyte-associated protein 4 has been shown to be involved in regulation of T cells activation.

The impaired ability to dispose of apoptotic material rapidly may increase the chance of an inappropriate immune response to this material. A deficit in clearance of apoptotic material through usual pathways may be explained by the presence of the classic LE cell observed in SLE. This represents granulocytes that have phagocytosed apoptotic debris (Schmidt-Acevedo et al, 2000). Deficiency in complement proteins has long been associated with SLE (Elliot and Mathieson, 1953). The reduction in early reacting components of the classical pathway may explain an inability to clear apoptotic material effectively as these proteins usually bind to apoptotic material and facilitate its clearance (Korb and Ahearn, 1997). An increase in the amount of apoptotic material may also
present a threshold beyond which an immune response is mounted. This may explain why a lupus flare often occurs after sun exposure or a viral infection as both these processes lead to an increase in apoptosis (Olson and Everett, 1975; Griffin and Hardwick, 1997).

In addition to abnormal levels apoptotic material, defects in the mechanisms of apoptosis may also play a role in the disturbance of immune control towards autoimmunity. The most common example being defects in the cell surface receptor, FasR, associated with the apoptosis signalling pathway and its cognate ligand, FasL. These observations were found in lupus prone mice later identified to have defective fas receptors (as in the MRL/lpr) or defective fas ligand (as in the MRL/gld) develop severe SLE like autoimmunity (Cohen and Eisenberg, 1991). Immune dysfunction including impairment of apoptotic processes is a complex subject with multiple links. For example, mice defective in Fas have been shown to have increased susceptibility to infection (Huang et al., 1998); certain viral infections have been associated with development of lupus and these infections in turn have also have been observed to have anti-apoptotic activities on immune system cells (Henderson et al., 1991).

Apoptosis may also play a role in exacerbating tissue damage, for example in glomerulonephropathy and cutaneous injury, which occurs as a result of the inflammatory condition associated with lupus. Here, the presence of autoantibody results in apoptosis of target cells by the process of antibody dependent cell mediated cytotoxicity and the presence of pro-inflammatory cytokines has been shown to increase fas mediated apoptosis in inflamed tissue (Lorz et al., 2000). If released into the circulation apoptotic material is procoagulant and may be involved in the vascular complications associated with SLE (Bombeli et al., 1997).

1.7.7 Cellular abnormalities and cytokine dysregulation

Several immune abnormalities have been found to be associated with SLE. Since immune function is very complex, determining whether these aberrations are a cause or consequence of the disease is an active area of research. Initial studies of immune function showed that suppressor T cell function was impaired in SLE (Kaufman et al., 1979). These cells, as described, are involved in the suppression of effector T cells and thus dysfunction may lead to increased inappropriate immune activity. It also now well accepted that SLE subjects have increased numbers of B cells (Blaese et al., 1980) and B cell activation is increased in many SLE subjects (Ginsburg et al., 1979). More recent work has focused on T cell and B cell receptor signalling in SLE and when activated, T cells showed significantly increased Ca\(^{2+}\) responses (Vassilopoulos et al., 1995) and enhanced production of
tyrosine phosphorylated cellular proteins, a T cell receptor (TCR) mediated biochemical event (Liossis et al., 1998); similar B cell receptor abnormalities have also been shown (Liossis et al., 1996). SLE subjects compared to normal subjects or subjects with other systemic autoimmune diseases SLE subjects were found to have TCRζ chain deficiency and this may play a role in signal-transducing abnormalities (Brundula et al., 1999).

The level of cytokines in SLE subjects has also been found to differ compared to healthy controls. In particular, an increase in IL-10 related to B cell hyperactivity (Llorente et al., 1995). The levels of cytokines produced by T helper cells involved in autoantibody production are also of interest in SLE as they signal the differentiation of T helper cells and affect the course of the immune response. Th1 cells secrete IFNγ, IL-2 and TNF-β and Th2 cells produce IL-4, IL-6, IL-10 and IL-13 (Mosmann et al., 1986). Th1 cells are mainly involved in activating macrophages and are sometimes called inflammatory CD4 T cells (Munoz Fernandez et al., 1992). Th2 cells are mainly involved in stimulating B cells to produce antibody and are often called helper CD4 T cells (Parker, 1993). Consistent with their effector function, the Th1-type response has been shown to be prevalent in the cell-mediated autoimmune disease insulin dependent diabetes mellitus (Monsann and Sad, 1996) and it has been suggested that Th2 cells may play an important role in the development of diseases such as SLE where the pathological response is mediated by autoantibodies (Goldman et al., 1991).

1.7.8 T helper cell cytokines in SLE

The role of cytokines in SLE has been investigated and, contrary to the theory that Th2 cytokines might dominate the immune response in this disease, there has been considerable evidence that IFNγ plays an important role and may be involved in the pathology of the disease. This was first suggested by Jacob et al. in 1987 who found that lupus mice receiving IFNγ had accelerated disease whilst those receiving anti-IFNγ antibodies at an early stage showed significantly delayed onset of disease. Later studies on the measurement of cytokine levels in SLE mice demonstrated that they had increased levels of IFNγ (Fan and Wuthrich, 1997) and experiments carried out with IFNγ overexpressing transgenic mice were shown to develop a T cell dependent SLE-like syndrome (Seery et al., 1997). Increased levels of IFNγ have been demonstrated in the serum of patients with SLE (al-Janadi et al., 1993) and in vitro experiments have shown higher levels of IFNγ expression in PBMC of SLE patients compared to controls after mitogenic stimulation (Gerez et al., 1997). In more recent studies however, measurement of IFNγ and IL-4 (the main Th1 and Th2 cytokines respectively) in stimulated peripheral blood T-cells, and unstimulated and stimulated whole blood T lymphocytes, of SLE patients showed no difference in the levels of these cytokines between SLE
normal controls (Nagy et al, 2000; Converso et al, 2000). Initial findings implicating Th1 cytokines in lupus immunopathology should also not rule out a role for Th2 cytokines. For example, studies have shown lupus mice have reduced mortality and disease when treated with anti-IL-4 antibody and the IL-4 receptor (Nakajima et al, 1997; Schorlemmer et al, 1995). The evidence that has been discussed here can be used to illustrate that the involvement of Th1 and Th2 cytokines in SLE is far more complex than the simplistic theory of Th1 and Th2-type responses and their known functions.

1.8 Breakdown of tolerance

In SLE many autoantibodies have been characterised as high affinity, eg autoantibody to Ro60 present in SLE sera is of predominantly IgG1 subclass (Maran et al, 1993). The production of high affinity antibody is a T cell dependent process involving antigen presentation to T helper cells via the MHC class II antigen presenting pathway (figure 1.3A) where extracellular antigens are taken up by antigen presenting cells and degraded by proteases into fragments for presentation to T helper cells (Cresswell, 1994). For autoreactive T cells to arise in the development of autoimmunity T cell tolerance must be impaired and there are many theories as to how this may occur.

T cell epitopes that are not normally recognised by T cells are termed cryptic epitopes. There may be reasons why these these epitopes may not normally be exposed to T cells (figure 1.3B). Firstly the antigen may be inaccessible to the immune system (figure 1.3Bi). Here tolerance may be broken by damage to cells exposing antigen with cryptic epitopes to the immune system for example, in the autoimmune condition, Good pasteur syndrome, reactive oxygen species have been shown to expose cryptic epitopes, which are normally located in a site of immune privilege to the immune system (Kalluri et al, 2000). Also viral infection may also lead to release of sequestered autoantigens through tissue damage (Miller et al, 1997). Secondly cryptic epitopes may be destroyed under normal circumstances during antigen processing (figure 1.3Bii). Tolerance may be broken here if antigen processing is altered, peptides released by protease cleavage may become altered possible resulting in the generation of cryptic epitopes. For example failure of glycosylation has indicated the generation of an autoantigenic peptide after proteolysis by granzyme B in Rasmussen’s encephalitis (Gahring et al, 2001). Finally peptides containing cryptic epitopes may not be presented due to a lower affinity for binding MHC molecules compared to those containing immunodominant epitopes (figure 1.3Biii). Again using glycosylation as an example, introduction of glycoslation at a peptide anchor residue required for effective MHC binding blocked the binding of the glycopeptide to the MHC molecule (Ishioka et al, 1992). Perhaps here, glycosylation suppresses the presentation of peptides containing cryptic epitopes. The above mechanisms demonstrate how cryptic epitopes
peptides containing cryptic epitopes. The above mechanisms demonstrate how cryptic epitopes might normally avoid T cell recognition; however there are several circumstances which may lead to the presentation of cryptic epitopes (figure 1.3C).

An increase in antigen concentration may lead to increased amount of cryptic epitope during processing such that it is displayed on the antigen presenting cell surface in quantities sufficient for T cell recognition (figure 1.3Ci). In SLE a defect in apoptosis has been demonstrated leading to an accelerated rate (Emelen et al, 1994) and since apoptotic cells are cleared by macrophages (Fadock et al, 1993) involving antigen presentation this may lead to presentation of cryptic epitopes through a mechanism involving increased antigen concentration. The effects of antibody binding prior to antigen uptake by antigen presenting cells may also lead to alterations in protease activity leading to increased display of cryptic epitopes (figure 1.3Cii), and mechanisms of receptor-mediated antigen uptake by Fc receptors may also effect alterations in antigen processing as shown in figure 1.3Ciii (Burkhardt et al, 1992; Lanzavecchia, 1995). Antigen may undergo extracellular degradation prior to antigen uptake and this will also affect the epitopes that are presented (figure 1.3Civ) for example, cartilage autoantigens implicated in rheumatoid arthritis are highly susceptible to degradation by extracellular proteases (Buttle et al, 1995).

Production of cryptic epitopes or the destruction of immunodominant epitopes may also depend on the processes occurring after activation of the antigen presenting cell. Here changes in proteolytic cleavage may be a major mechanism leading to the production of cryptic epitopes. Cellular damage may result in changes to antigen structure leading to altered proteolytic processing of peptide antigen. For example, proteolytic cleavage of self antigen following xenobiotic-induced cell death has been shown to produce a fragment with novel immunogenic properties (Pollard et al, 2000) and fragmentation of proteins by ROS damage may generate autoantigens in systemic sclerosis (Casicola-Rosen et al, 1997). Although the mechanism of autoantigen generation not been investigated here it may involve altered antigen processing of the modified antigens (figure 1.3Cv). Finally the type of antigen presenting cell whether, B cell, macrophage or dendritic cell may represent different pathways of antigen presentation. In certain autoimmune conditions there may be impairment in one particular pathway leading to presentation via an alternative pathway leading to changes in the way antigen is processed. In addition the pathway of antigen presentation may differ within the same antigen presenting cell (figure 1.3Cvi), for example the antigen processing of two major T-cell epitopes on Streptococal M5 protein have been shown to occur in two different endosomal compartments (Delvig and Robinson, 1997).
Figure 1.3: Antigen uptake, processing and presentation; why some epitopes may be cryptic adapted from Warnock and Goodacre, 1997. Antigen uptake, processing and presentation (A) where by extracellular antigens are taken up by antigen presenting cells and degraded by proteases into fragments and epitopes are presented to T helper cells. T cell epitopes that are not normally recognised by T cells are termed cryptic epitopes and there may reasons why cryptic epitopes may not normally be exposed to T cells (B). The antigen may be inaccessible to the immune system (i), may be destroyed under normal circumstances during antigen processing (ii) or peptides containing cryptic epitopes may not be presented due to a lower affinity for binding MHC molecules compared to those containing immunodominant epitopes (iii). There are several ways in which cryptic epitopes may be revealed for T-cell recognition (C). An increase in antigen concentration may lead to increased amount of cryptic epitope during processing such that it is displayed on the antigen presenting cell surface in quantities sufficient for T cell recognition (i). The effects of antibody binding prior to antigen prior to uptake by antigen presenting cells may also lead to alterations in protease activity leading to increased display of cryptic epitopes (ii), and mechanisms of receptor-mediated antigen uptake by Fc receptors may also effect alterations in antigen processing (iii). Antigen may undergo extracellular degradation prior to antigen uptake and this will also affect the epitopes that are presented (iv). Production of cryptic epitopes or the destruction of immunodominant epitopes may also depend on the processes occurring after activation of the antigen presenting cell (v) and finally the type of antigen presenting cell whether, B cell, macrophage or dendritic cell may represent different pathways of antigen presentation (vi).
Normal processing of antigen may also still result in a breakdown of tolerance at the level of T cell recognition, for example, autoreactive T cells may be activated due to the structural similarity between self-antigen and microbial peptide, a mechanism referred to as molecular mimicry (Fujinami et al, 1983). With similar amino acid sequences self-peptide and microbial peptides have been shown to activate T cell clones (van Eden et al, 1985).

1.9 T cell and B cell epitopes in SLE
The identification of T cell and B cell epitopes of SLE autoantigens is a prominent area of research in SLE. The B cell epitopes have been more readily studied as autoantibody can be used to detect which peptides of an autoantigen recognise autoantibody. Using short peptides from Ro60 and a panel of anti-Ro positive sera as many as 20 B cell epitopes have been identified (Scofield et al, 1991). While Ro autoantibodies in some sera were found to bind many epitopes, other sera had Ro autoantibodies that only recognised a few. Studies of Ro autoantibodies which bind peptide fragments of Ro have been shown to be part of the population of autoantibodies which bind to native Ro (Huang et al, 1995) and this agrees with the findings that autoantibodies to the Ro60 have been demonstrated to be mainly directed to the native protein and are conformation dependent (Itoh et al, 1991). Many groups have investigated the B cell epitopes of Ro60 using either synthetic peptides or recombinant antigen with methods for blotting, ELISA or immunoprecipitation and the findings are summarized in figure 1.4. The number of subjects and sera included differed in these investigations and so did parameters on which subjects were selected. The main conclusion of the comparison of the different studies is that despite different experimental approaches a consistent finding is a major antigenic region (shown in black recognised by a high proportion of sera) in the middle of Ro60 (within residues 155-326). This is a very hydrophilic region of the protein and is suggested to be an exposed part of the protein and therefore these epitopes may be part of the population, which bind to the native Ro. Less consistently found are epitopes recognised by an intermediate number of sera are (shown in dark grey) and minor epitopes recognised by a small percentage of sera (shown in light grey).

T cell epitopes have been less well characterised but may be of more importance in the identification of potential initiating autoantigens in the disease. The activation of T helper cells is an initial event in the activation of the autoimmune response and for B cell autoantibody production the specificity of antigen to antibody changes during maturation of the immune response. T cell studies have investigated the autoimmune response to dsDNA and nucleosome, two of the more common autoantibodies in SLE. A peptide-induced model of SLE, where the peptide was a mimetope of
dsDNA on a branched polylysine backbone, was shown to which induce a SLE-like syndrome in nonautoimmune mice. T cell proliferation and autoantibody production were shown to require the presence of both the peptide and the polylysine backbone. The breakdown in tolerance was suggested to arise through activation of T cells to foreign antigen and molecular mimicry at the B cell level as the T cells responding to a foreign antigen did not cross react with peptides from self antigen (Khalil et al, 2001). Another study identified a minimal T cell epitope recognized by antinucleosome Th cells in the C-terminal region of histone H4. Here multiple T epitopes were identified after immunization of nonautoimmune mice with adjoining peptides covering the whole sequence of histone H4 (Decker et al, 2000).

Figure 1.4: Representation of B cell epitopes of 60kDa Ro60 adapted from Wharen-Herlenius et al, 1999. B cell epitopes described based on several different methods using either synthetic peptides or recombinant antigen with methods for blotting, ELISA or immunoprecipitation. The number of subjects and sera included differed in these investigations and so did parameters on which subjects were selected. Despite different experimental approaches (a-f) the position of a major antigenic region in the middle of Ro60 shown in black (within residues 155-326) appears consistent. This is a very hydrophilic region of the protein and is suggested to be an exposed part of the protein. Those epitopes recognised by less of the sera are shown in dark and light grey respectively. The position RNP-80 and zinc finger structural features of Ro60 are also shown.
Exposure to UV

The observation that autoantigens targeted in SLE were clustered in two populations of surface blebs on keratinocytes cells dying from apoptosis suggested a mechanism for how normal cellular antigens could become exposed to the immune system (Cascicola-Rosen et al, 1994), cellular antigen with in these surface blebs also targets for damage by extracellular insults. A major symptom of SLE is an unusual reaction to sunlight resulting in a photosensitive skin rash (Cohen and Isenberg, 1996) and therefore the role UV in sunlight plays in the pathogenesis of the autoimmune disease SLE has often been questioned. Associations between cellular damage caused by UV in sunlight and disease are well known, for example in the link between sunburn and the risks of developing skin cancer, where studies show that UVB is readily absorbed by the skin and is known to cause cancer in mammals (Tyrell and Pidoux, 1987). Much research has been carried out into the mechanism of UV light induced damage to the eye and it has been shown that UVB is capable of inducing protein modifications of the eye lens proteins and these are thought to be involved in the formation of cataracts (Spector, 1995). UV in sunlight may initiate modification to Ro60 and other autoantigens in SLE. Skin lesions occur in SLE that are exacerbated by UV light and photosensitivity has been positively associated with the presence in serum of anti-Ro60 antibodies (Mond et al, 1989). It is possible that in SLE photosensitivity reactions occurring in the cells of the skin may result in modification of proteins or other biomolecules which has implications for the development of autoimmunity.

UV damage to the skin is thought to involve only UV-A (320nm-400nm) and UV-B (290-320nm); UV-C (100-290nm) is filtered by the O$_3$ layer and therefore appears to have little biological significance (Gasparro and Brown, 2000). Some of the damage induced is caused by absorption of UV by skin photosensitzers capable of generating reactive species. DNA is able to absorb UV-B leading to the formation of pyrimidine hydrates or pyrimidine crosslinks (Carell et al, 1995) and it is also possible that proteins may be able to act as photosensitzers or become damaged by the reactive species generated. ROS production within human keratinocyte cell lines has been shown following UV-B irradiation using an intracellular ROS probe (Lawley et al, 2000) and this may be a mechanism whereby SLE photosensitivity reactions occurring in the cells of the skin may result in modification of proteins or other biomolecules which has implications for the development of autoimmunity.
1.11 **ROS induced oxidative damage**

In SLE, elevated levels of ROS may also result from the activation of inflammatory cells during the immune response (PithonCuri et al, 1998) and these may contribute to cellular oxidative stress with potential to cause oxidative modification to protein that could in turn lead to generation of antigenic structures (Utz and Anderson, 1998). From inflammatory cells ROS are released during phagocytosis by a respiratory burst where $O_2^-$ is generated from the reduction of $O_2$ (Babior et al, 1997). Further ROS are formed when $H_2O_2$ mainly formed from the dismutation of $O_2^-$ ($O_2^- + O_2^- \rightarrow 2H^+ \rightarrow H_2O_2 + O_2$) is used by myeloperoxidase to make HOCl (Kettle and Winterbourn, 1997). ROS are also generated within cells as a result of normal cellular metabolism where the major source of the ROS is through leakage of electrons from the mitochondrial electron transport chain generating superoxide ($O_2^-$) (Turrens, 1997). In addition several oxidoreductase enzymes, for example NADPH oxidase (Henderson and Chappell, 1996), xanthine oxidase (Kehrer et al, 1987) have been reported to reduce $O_2$ to $O_2^-$. The activities of the cytochrome P450 enzymes (Goepfar et al, 1995) and cyclooxygenases are also known to generate ROS (Capdevila et al, 1995).

Several ROS which are highly unstable and reactive chemical species are generated during oxidation reactions often catalyzed by transition metal ions. The term ROS is used to define not only oxygen radicals such as superoxide ($O_2^-$), hydroxyl (OH'), peroxyl (RO''), alkoxyl (RO') and hydroperoxyl (HO'') but also non-radical derivatives of $O_2$ such as hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), ozone (O$_3$), singlet oxygen and peroxynitrite (ONOO'). The ability of transition metal ions to accept/donate single electrons means they are also powerful catalysts of free-radical reactions (Halliwell and Gutteridge, 1984). Iron and copper are involved in the generation of OH' a major radical in biological systems by the Haber-Weiss reaction, first suggested by Weiss in 1935 (Halliwell and Gutteridge, 1990). The iron-catalyzed and copper-catalyzed Haber-Weiss reaction is shown in figure 1.5.
Fe$^{3+}$ + O$_2^-$ → Fe$^{2+}$ + O$_2$

Fe$^{2+}$ + H$_2$O$_2$ → OH$^-$ + OH$^-$ + Fe$^{3+}$

or

Cu$^{2+}$ + O$_2^-$ → Cu$^+$ + O$_2$

Cu$^+$ + H$_2$O$_2$ → OH$^-$ + OH$^-$ + Cu$^{2+}$

Net: O$_2^-$ + H$_2$O$_2$ → O$_2$ + OH$^-$ + OH$^-$

Figure 1.5: Iron-catalyzed and copper-catalyzed Haber–Weiss reaction adapted from Halliwell and Gutteridge, 1990. The net equation shows the formation of the hydroxyl free radical as the product of the reaction between superoxide and hydrogen peroxide as originally proposed by Haber and Weiss. This was later found to occur in two steps known as Fenton chemistry and is catalysed by transition metal ions. In the first reaction superoxide reduces transition metal ion and then in the second reaction the reduced metal ion then reacts with hydrogen peroxide to produce the hydroxyl radical.

Under conditions of cellular oxidative stress, where ROS are present at elevated levels, oxidative damage to several cellular biomolecules may be initiated and major targets of oxidative attack that have been extensively studied are protein (Tamarit et al, 1998), DNA (Breen and Murphy, 1995) and lipid (Barber and Thomas, 1978). Under normal cellular conditions the prooxidant capacity of reactive oxygen species is counter-balanced by a complex array of antioxidant defences which are present to protect the cells of the body from oxidative damage. These include antioxidant enzymes which catalytically remove reactive oxygen species, for example superoxide dismutase (Fridovich et al, 1995) and catalase (Winterbourne and Stern, 1987), low-molecular mass agents that scavenge reactive oxygen species, some of which are obtained from the diet for example, ascorbic acid (Barja et al, 1994) and α-tocopherol (Stephens et al, 1997) and also proteins that minimise availability of pro-oxidants such as caeruloplasmin which binds copper ions which are powerful catalysts of free radical damage (Gutteridge et al, 1985).
1.12 Consequences of oxidative damage

The consequence of accumulating damage to biomolecules is to result in cellular dysfunction and perhaps development of disease (Berlett and Stadtman, 1997). ROS have been implicated in the pathology of several diseases. Some examples are: ageing related diseases, for example cataract formation has been associated with the accumulation of oxidised protein in the lens of the eye (Andley et al, 1987), atherosclerosis where oxidation of LDL (resulting in abnormal uptake of LDL by macrophages resulting in foam cell formation) which has been shown to involved in the development of atherosclerosis (Steinberg et al, 1989), diabetes where glucose derived oxidising species have been found as a result of high levels of plasma glucose (Krapfenbauer et al, 1998), neurodegenerative diseases such as Alzheimer's disease where elevated levels of oxidised protein has been reported (Smith et al, 1991) and autoimmunity for example rheumatoid arthritis where oxidised IgG has been has been suggested to lead to chronic inflammation in the affected joint (Lunec et al, 1985).

ROS induced damage has been suggested as a mechanism leading to the development of autoimmunity. Posttranslational modification of protein, including ROS induced changes, has been reviewed as a mechanism whereby changes in the antigen structure may lead to self antigen being recognized as foreign (Utz and Anderson, 1998). Experimental evidence supporting this theory was described by Wuttge et al in 1999 who demonstrated that T cell tolerance to self proteins in mice could be broken by immunization with homologous protein modified using reactive aldehydes which are known to be generated during lipid peroxidation in cells. In other examples modifications to proteins caused by reactive oxygen species have been associated with autoimmune disease, for example in the autoimmune disease Scleroderma, autoantigens have been shown to be uniquely fragmented in the presence of ROS and specific metals (Casciola-Rosen et al, 1997). Also, more recently, a ROS generated aggregate of a major autoantigen in type 1 diabetes mellitus has been shown to be predominantly recognised using serum antibodies from these patients (Trigwell et al, 2001).

1.13 Protein Oxidation

Oxidising Sources

Protein molecules are a major target for reactive oxygen species (Gieseg et al, 2000) leading to oxidative modifications of protein structure. In addition to ROS, UV light, present in the sun’s rays, has also been described as an agent capable of inducing protein oxidation either by direct absorption of UV light by protein (Gehardt et al, 1999) or by the generation of ROS intermediates
(Balasubramanian et al, 1990). Also lipid peroxidation, resulting from ROS damage to lipids leads to the generation of reactive aldehyde products and these too are able to react with protein molecules thus modifying their structure (Fogalman et al, 1980). Unlike DNA and lipids where specific biomarkers of damage have been identified, the products of protein oxidation include various amino acid and structural modifications making protein oxidation difficult to quantify.

**Initiation of oxidation**

Initial pioneering studies on the oxidation of proteins by ROS involved exposing proteins to high energy radiation under conditions where the oxidising species were O$_2^-$, OH$^-$ or a combination of both species (Swallow, 1960; Garrison et al, 1962; Schuessler et al, 1984). It was demonstrated that the initiating species was OH$^-$ but the availability of O$_2$ and O$_2^-$ or its protonated form HO$_2^-$ determined the course of the oxidation process leading to modification to amino acid side chains, formation of protein-protein cross-linking reaction and to cleavage of peptide bonds. Using this system, Davies et al, 1987, catalogued a series of major modifications common to a wide variety of proteins and studied these modifications in further detail using BSA as a representative model protein. These included aggregation, fragmentation, changes in net electrical charge, loss of tryptophan and formation of dityrosine.

Radical-mediated tissue damage has been shown to occur in cells exposed to high oxygen tensions (Starke et al, 1987). Reperfusion and ischemia; and conditions of inflammatory stress were demonstrated as events leading to the elevation of ROS (Zweir et al, 1989; Weiss, 1989) and OH$^-$ was shown to cause oxidative modification of proteins by mixed function oxidation using several different enzymatic and nonenzymic metal ion-catalyzed oxidation (MCO) systems (Stadtman and Wittenberger, 1985; Deshpande and Joshi, 1985) The reaction was shown to proceed by the Haber-Weiss reaction as described in section 1.5 where H$_2$O$_2$ and reduced metal ion were generated from an electron donor in the mixed function oxidation system or by one-electron transfer to O$_2$ to form O$_2^-$ The demonstration that metal catalyzed oxidation caused oxidation of only one of several oxidation susceptible proteins situated at a probable protein metal binding site (Farber and Levine, 1986) and that oxidation induced inactivation of enzyme activity was insensitive to inhibition by free radical scavengers (Levine et al, 1981) lead to the hypothesis that metal catalysed oxidation was a site specific process occurring at the site of metal binding to the protein.
Oxidation of the protein backbone

Initial studies on the radiolysis of protein lead to mechanistic models for the oxidation reactions initiated by OH' (figure 1.6a). The protein backbone is a target of oxidative attack. OH initiates the reaction by causing loss of a hydrogen atom from the alpha carbon of an amino acid residue resulting in the generation of a carbon centred, alkyl radical (R') (figure 1.6b). This is then able to react with O₂ to forming a alkylperoxyl radical RO₂ (figure 1.6c). The following reactions mediated by HO₂⁻ or catalyzed by metal ions may form alkylperoxide (ROOH) (figure:1.6d) leading to the formation of an alkoxyl radical (RO) (figure 1.6e) and OH protein derivatives (figure 8f). Radicals produced in this chain of events may form new carbon centred radicals on a different protein or undergo reactions with other amino acids of the same protein. The carbon centred radical may react with another carbon centred radical leading to the formation protein-protein cross-links, in the absence of O₂ the formation of the alkoxyl radical may lead to peptide bond cleavage by diamide or α-amidation pathways (Figure 1.7).

Formation of protein carbonyls

Metal catalyzed oxidation and other oxidising sources have been show to induce the formation of carbonyl groups (Amici et al, 1989; Shacter et al, 1995; Silvester et al, 1998). Several amino acid residues, for example arginine and proline residues, undergo oxidation reactions to generate γ-glutamylsemialdehyde, and lysine residues are oxidised to amino-adipicsemialdehyde (Climent et al, 1989; Amici et al, 1989). Carbonyl groups may also be introduced into proteins, for example, the products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (which contain carbonyl groups) are able to react with lysine residues. Also glucose oxidation and glycation may produce carbonyl derivatives capable of reacting with amino acid residues (Degenhardt et al, 1998; Hunt et al, 1994). Therefore carbonyl formation is often used as a measure of protein oxidation under various conditions and an increase in carbonyl formation has been associated with the ageing process and certain disease states. (Oliver et al, 1989; Chapman et al, 1989; Smith et al, 1998).
Figure 1.6: Reactions of hydroxyl initiated oxidation of the protein backbone adapted from Berlett and Stadtman, 1997. Radiolysis of water results in the formation the hydroxyl radical (figure 1.6a), this can initiate oxidative attack on the protein backbone where loss of a hydrogen atom from an alpha carbon of an amino acid residue results in the generation of a carbon centred radical (figure 1.6b). This can react with oxygen to form an alkylperoxyl radical (figure 1.6c). Further reactivity with hydroperoxyl radicals and transition metal ions may result in formation of alkylperoxides (figure 1.6d), alkoxyl radicals (figure 1.6e) and hydroxyl protein derivatives (figure 1.6f).

Figure 1.7: Diamide (A) and α-amidation (B) pathways of peptide bond cleavage adapted from Berlett and Stadtman, 1997. In the diamide pathway of peptide bond cleavage the product of the N terminal end of the peptide is two amide groups as a diamide structure and the C terminal an isocyanate structure. In the α-amidation pathway of peptide bond cleavage the product of the N terminal end of the peptide is an amide group and the C terminal an N-α-ketoacetyl derivative.
Measures of protein carbonyls are a well established way of detecting protein oxidation (Levine et al, 1990; Buss et al, 1997; Robinson et al, 1999). Most assays involve the derivatisation of the carbonyl group with dinitophenylhydrazine leading to the formation of a stable dinitrophenylhydrazone product as illustrated in figure 1.8. The dinitrophenyl group absorbs UV at 370nm and hence carbonyls can be quantified by spectrophotometric methods. The recent development of antibodies to dinitrophenol has enabled detection of protein carbonyls by immunochemical methods as shown in the schematic diagram in figure 1.9. Immunochemical techniques provide significantly more sensitivity and specificity than other approaches despite the blotting technique being semi-quantitative.

**Figure 1.8:** Reaction of 2,4-dinitrophenylhydrazine with a carbonyl group; adapted from Stadtman, 1990. The carbonyl group may be an aldehyde with a single hydrogen atom attached to the carbonyl carbon or a ketone group with two hydrocarbon groups. The carbonyl group reacts with hydrazine group the 2,4-dinitrophenylhydrazine to form a hydrazone (2,4-dinitrophenylhydrazone) with the elimination of H$_2$O.

**Oxidation of amino acid side chains**

There are numerous oxidative modifications generated on amino acid side chains by various oxidising sources and some of the more commonly detected adducts are outlined here. The thiol groups in cysteine and methionine residues make them highly susceptible to oxidation. The initial product of methionine oxidation is methionine sulphoxide which can be further oxidised to sulphone. Cysteine residues are oxidised to disulphides (Amis 1979; Kopoldova et al, 1967; Purdie, 1967). The amino acids valine and leucine were shown to form protein hydroperoxides after addition of OH$^-$ in the presence of O$_2$ (Fu and Dean, 1997; Fu et al, 1995) and also the aromatic residue tryptophan was shown to undergo oxidation reactions leading to fluorescent oxidation products N-formylkynurenine and kynurenine (Finley et al, 1998). Other aromatic residues are sensitive to oxidation, many tyrosine oxidation products have been identified, for example by reactions with OH$^-$, ONOO$^-$, and HOCI leading to dihydroxyphenylalanine (DOPA), nitrotyrosine and chlorotyrosine respectively (Geiseg et al, 1993; Eiserich et al 1996). Alternatively, tyrosine radicals may be generated which may undergo cross-linking reactions to give dityrosine (Heinecke
Histidine undergoes oxidation reactions and has shown to be highly susceptible to reactions with singlet oxygen compared to other aromatic amino acids (Merkel and Kearns, 1972). Oxidation forms 2-oxohistidine (Lewisch and Levine, 1995) and other reactions involved in protein crosslinking (Balasubramanian et al, 1990).

**Figure 1.9:** Immunological approach for using DNPH to detect protein carbonyls, adapted from Shacter, E. (2000). Carbonyl groups are first derivatised with DNPH and then detected using an anti-DNP rabbit antibody. The primary antibody detects DNP labelled molecules, in this case DNP labelled protein. This can then be detected using an anti-rabbit HRP labelled secondary antibody and HRP activity can then be measured by chemiluminescence's detection.

### 1.14 Detection of protein oxidation

The large number of protein oxidation modifications has lead to the development of numerous assays for the detection of protein oxidation products. Some of the methods used for the detection of oxidative protein modifications are described. Electrophoresis (reducing and non-reducing) is commonly used for the detection of cross-links, aggregates and fragments and for the formation of disulphide bonds (Davies and Delsignore, 1987). Methionine sulfoxide may be detected by CNBr cleavage followed by amino acid analysis (Maier et al, 1995). Amino acid analysis has also been used for the detection of 2-oxohistidinol (Lewisch and Levine, 1995). Methods for carbonyl detection have been extensively studied as previously described (section 1.13). Instrumental techniques, for example HPLC and GCMS have been employed for the detection of many oxidation products for example dityrosine and tryptophan oxidation products which have fluorescent properties (Leewnburgd et al, 1998; Finley et al, 1998). These techniques have also been used for the detection of protein hydroperoxides, chlorotyrosine and nitrotyrosine products (Fu and Dean, 1997; Hazen...
and Heinecke, 1997; Shigenaga, 1997). For protein oxidation the measurement of more than one biomarker is often necessary in order to gain an understanding of the nature of the oxidizing species and oxidised products implicated in the pathology of specific diseases.
1.15 Aims of this thesis

- Overall the aim of the thesis was to investigate if oxidative modification of ‘self’ protein by pathological ROS may represent a mechanism whereby ‘self’ ‘protein is no longer recognised as ‘self’ by the immune system. Ro60 was chosen as a model autoantigen in the prototype autoimmune disease SLE and is a likely candidate of oxidative modification.

- The first specific aim was to obtain a source of purified recombinant human Ro60.
- To enhance the solubility of overexpressed Ro60 by the attachment of a highly soluble fusion partner and also to aid in the subsequent purification of Ro60 by affinity chromatography by subcloning Ro60 (available in the expression vector pET8c for the production of recombinant Ro60 protein) into expression vector pMAL for the production of a maltose binding protein fusion protein.

- The second aim was to investigate oxidative damage to Ro60 in vitro.
- UVB exposure and ROS generated by MCO were used as sources of oxidation to achieve oxidative modification of Ro60.
- After oxidation the aim was to detect products of protein oxidation, aggregation, fragmentation and carbonyl formation after Western blot and dot blot procedures using anti-Ro and anti-DNP (for carbonyl detection) antibodies.
- Further aims were to investigate whether damage was dose responsive and whether different antioxidants could attenuate oxidative damage in order to elucidate potential mechanisms of oxidation.

- The final aim of the thesis was to investigate if Ro60 modified by ROS possessed enhanced immunogenicity in PBMC cultures from Ro positive subjects.
- Since the immune response to Ro60 in SLE is T cell dependent the aim was to establish methodology for the detection of IFN and IL-4 secretion, the two major T cell cytokines released after T cell activation using magnet assisted cell separation (MACS) cytokine secretion assay with flow cytometry detection.

- The aim was to combine these experiments with a flow cytometry method already established for annexin V and propidium iodide (PI) detection to look at cell death responses after cell stimulation, which may also be altered during response to autoantigen.
CHAPTER 2:

MATERIALS AND METHODS
2.0 Materials and Methods

2.1 Materials

2.1.1 Production of Ro60 maltose binding protein fusion protein

Bacterial Strains

E. coli BL21(DE3)pLysS+pET8c/Ro60 was a gift from Dr. G. Pruijn, University of Nijmegen, The Netherlands. E. coli TB1+pMAL, E. coli TB1 and E. coli PR745 were kindly provided as glycerol stocks by Dr. C. Hewitt, Centre for Mechanisms of Human Toxicity, University of Leicester, U.K.

E. coli culture

DIFCO Tryptone, yeast extract and agar were purchased from BD Biosciences, Oxford, UK and sodium chloride was from Fisher, Loughborough, UK. Chloramphenicol and ampicillin and glycerol were from Sigma, Poole, UK. Petri dishes were from Bibby Sterilin Ltd, Staffordshire, UK

Plasmid preparation (Lithium chloride and plasmid mini-preps)

The following reagents were from Sigma: Trizma-base, ethylenediaminetetraacetic acid (EDTA), glucose, sodium hydroxide, sodium dodecyl sulphate (SDS), potassium acetate, isopropanol, lithium chloride, RNase A, polyethylene glycol (PEG), sodium chloride, sodium acetate, phenol, and chloroform. Absolute ethanol and glacial acetic acid were from Merck Ltd, Lutterworth, UK. Hydrochloric Acid (HCl) was from Fisher.

Agarose Gel Electrophoresis

Trizma-base, boric acid, EDTA, agarose, ethidium bromide and gel loading solution Type 1, 6× concentrate containing 0.025%(w/v) bromophenol blue, 40%(w/v) sucrose, 0.1M EDTA and 0.5%(w/v) SDS were from Sigma. 500bp ladder was purchased from GibcoBRL Life Technologies, Paisley, UK.

Restriction digestion

Restriction enzymes and restriction digest buffers were from New England Biolabs, Hertfordshire, UK. These were as follows: NcoI and BamH1 with BamH1 digest buffer containing 150mM sodium chloride, 10mM Tris-HCl, 10mM magnesium chloride and 1mM dithiothreitol (DTT), pH 7.9, and EcoR1 and XbaI with EcoR1 buffer containing 50mM sodium chloride, 100mM Tris-HCl, 10mM Magnesium chloride, 0.025% Triton X-100 and 100µg/ml BSA, pH 7.5. XbaI digest buffer
contained 50mM sodium chloride, 10mM Tris-HCl, 10mM magnesium chloride, 1mM DTT and 100μg/ml BSA, pH 7.9. NucleoTrap Nucleic Acid Purification Kit was purchased from Clontech Laboratories UK Ltd, Hampshire, UK.

**PCR amplification**

Native Pfu DNA Polymerase and Pfu polymerase buffer containing 100mM Tris-HCl, 20mM magnesium chloride, 100mM potassium chloride, 60mM ammonium sulphate, 1%(v/v) Triton X-100 and 100μg/ml bovine serum albumin (BSA), pH 8, were purchased from Stratagene Europe, The Netherlands. PCR primers were synthesised by the Protein and Nucleic Acid Laboratory, University of Leicester, U.K and re-precipitated using absolute ethanol (Merck Ltd) and sodium acetate from Sigma. Deoxynucleotide triphosphate (dNTP) mix and magnesium chloride were from GibcoBRL Life Technologies.

**Ligation Reactions**

T4 DNA ligase and T4 DNA ligase buffer containing 250mM Tris-HCl, 50mM magnesium chloride, 5mM adenosine triphosphate, 5mM DTT and 25% (w/v) PEG was purchased from GibcoBRL Life Technologies.

**Competent cells**

All chemicals were from Sigma: potassium acetate, manganese chloride, potassium chloride, calcium chloride, glycerol and 3-(N-morpholino) propane sulfonic acid (MOPS). LB broth was from Scientific Laboratory Supplies and ampicillin was from Sigma.

**Bacterial Transformations**

LB broth was from Scientific Laboratory Supplies and ampicillin was from Sigma.

**Small scale overexpression of Ro60 maltose binding protein fusion protein**

LB broth was from Scientific Laboratory Supplies. Ampicillin, glucose, Trizma-base and EDTA were from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Bio Gene Ltd, Cambridge, UK.
**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**
Acrylamide:bisacrylamide 29:1 and precision prestained protein standards were from Biorad, Hertfordshire, UK. Electrophoresis grade Trizma-base, glycine, SDS, glycerol, bromophenol blue, DTT, ammonium persulphate (APS), N, N, N,' N'tetramethylethylenediamine (TEMED) were purchased from Sigma.

**Coomassie Blue Staining**
Coomassie brilliant blue R250 was purchased from Sigma. Glacial acetic acid was from Merck Ltd and methanol was from Fisher.

**Cell lysis**
Trizma base, sodium chloride, EDTA and phenyl methane sulphonyl fluoride (PMSF) were from Sigma; and the Bio-Rad protein assay was from BioRad. Concentrated hydrochloric acid was from Fisher.

**Affinity purification**
Trizma base, sodium chloride, EDTA and PMSF, and maltose were from Sigma. Amylose resin was from New England Biolabs and Centricon centrifugal filter devices were from Amicon, Gloucestershire, UK.

**Factor Xa cleavage**
Factor Xa was from New England Biolabs and Microcon centrifugal filter devices were from Amicon.

**Isolation of inclusion bodies**
Trizma-base, sodium chloride DNAse, lysozyme, Nonidet P-40 (NP-40), EDTA, Triton X-100 and urea were purchased from Sigma. Oakridge centrifuge tubes were from Nalgene Labware, Hereford, UK.

**2.1.2 Purification of Ro60 (without MBP fusion)**
LB broth was from Scientific Laboratory Supplies. Ampicillin, chloramphenicol, glucose, Trizma-base, sodium chloride, EDTA, DNAse, lysozyme, NP-40, sucrose, guanidine hydrochloride and phosphate buffered saline (PBS) in tablet form were from Sigma. IPTG was purchased from Bio Gene Ltd. Zwittergent-3.14 was from Calbiochem, Nottingham, UK.
**Silver Staining of SDS-PAGE gels**

Glacial acetic acid was from Merck Ltd. Methanol and silver nitrate were from Fisher. Sodium carbonate and formaldehyde were from Sigma.

**Western Blot Analysis**

Methanol and hydrochloric acid were from Fisher. Marvel dried skimmed milk powder was purchased locally. All other chemicals were from Sigma: Trizma-base, glycine, sodium chloride, polyoxyethylenesorbitan monolaurate (Tween 20). Polyvinyl difluoride (PVDF) transfer membrane was also from Sigma. 3mm filter paper was from Whatman International Ltd, Maidstone, UK. Enhanced chemiluminescence (ECL) Western blotting detection reagents and high performance chemiluminescence film were purchased from Amersham Life Science, Buckinghamshire, UK. Anti-Ro60 antibodies were from Serologicals Corporation, Oxford, UK and anti-human IgG horseradish peroxidase (HRP) conjugate was from Sigma.

**Concentration of Ro60**

The following items were purchased from Sigma: dialysis tubing with a flat width of 76mm, an average diameter of 49mm and a molecular weight cut off of 12,000Da, PBS, polyethyleneglycol of molecular weight 8000Da and sodium sulphite. Concentrated sulphuric acid was from Fisher.

**Protein Assays**

The Bio-Rad protein assay reagent and the Micro BCA protein assay reagent kit were purchased from Bio-Rad and Perbio Science UK Ltd, Cheshire, UK respectively. Bovine serum albumin (BSA) was from Sigma.

**2.1.3 UV and ROS induced damage of Ro60**

**Treatment of Ro60 with pro/anti-oxidants**

30% (w/w) hydrogen peroxide solution, copper (II) sulphate, mannitol, dimethyl sulphoxide (DMSO), desferrioxamine (DFO) and diethylenetriaminepentaacetic Acid (DTPA) were from Sigma.

**ROS treatment of cell lysates**

**Maintenance of NDF cell lines**

Normal dermal fibroblasts were isolated from healthy adult human skin by Angie Gillies, Department of Pathology, University of Leicester, UK. Dulbecco’s Modified Eagles Medium with
GlutaMAX™-1, 1000mg/L glucose and sodium pyruvate (DMEM) and Dulbecco’s phosphate buffered saline (DPBS) were purchased from Sigma. Other reagents were foetal calf serum from Labtech International, East Sussex, UK, GlutaMAX™-1 from Invitrogen Ltd, Paisley, UK, trypsin EDTA solution from GibcoBRL Life Technologies and gentomycin from Sigma. Cell culture flasks (175cm²) were from Greiner Bio-one, Gloucestershire, UK.

**Preparation of cell lysate**

All reagents were purchased from Sigma: antipain, chymostatin, EDTA, leupetin, magnesium acetate, β-mercaptoethanol, pepstatin A, potassium chloride and Trizma-base.

**Pro-oxidant treatment of cell lysate**

30% (w/w) hydrogen peroxide solution and copper (II) sulphate were purchased from Sigma.

**Dot Blotting**

Methanol and hydrochloric acid were from Fisher, Marvel dried skimmed milk powder was purchased locally. All other chemicals were from Sigma: Trizma-base, glycine, sodium chloride, and Tween 20. Pure nitrocellulose was purchased from Bio-Rad. 3mm filter paper was from Whatman International Ltd. ECL Western blotting detection reagents and high performance chemiluminescence films were purchased from Amersham Life Science. Anti-Ro60 antibodies (item I.D. FT 11008854) were from Serologicals Corporation and anti-human IgG HRP conjugate from Sigma.

**Protein carbonyl dot blot assay**

Methanol and hydrochloric acid was from Fisher, Marvel dried skimmed milk powder was purchased locally. All other chemicals were from Sigma: Trizma-base, glycine, sodium chloride, Tween 20 and dinitrophenylhydrazine (DNPH). Pure nitrocellulose was purchased from Bio-Rad and 3mm filter paper from Whatman International Ltd. ECL Western blotting detection reagents and high performance chemiluminescence film were purchased from Amersham Life Science. Polyclonal (rabbit IgG) antibody raised against dinitrophenyl conjugated to keyhole limpet hemacyanin (anti-DNP-KLH) was from Molecular Probes, Cambridge, UK and anti- rabbit IgG-HRP was from Sigma.
**Protein Carbonyl Western blot Assay**
Methanol and hydrochloric acid was from Fisher, Marvel dried skimmed milk powder was purchased locally. All other chemicals were from Sigma: Trizma-base, glycine, sodium chloride, Tween 20 and DNPH. PDVF transfer membrane was also from Sigma. 3mm filter paper was from Whatman International Ltd. ECL Western blotting detection reagents and high performance chemiluminescence films were purchased from Amersham Life Sciences. Polyclonal anti-DNP-KLH (rabbit IgG) antibody was from Molecular Probes, Cambridge, UK and anti-rabbit IgG-HRP from Sigma.

### 2.1.4 Cell Activation and Cell Death of PBMCs treated with Ro60

**Isolation of peripheral blood mononuclear cells**
DPBS, Histopaque-1077 and EDTA were purchased from Sigma.

**Treatment of PBMC with antigen in vitro**
Roswell Park Memorial Institute Medium 1640 (RPMI) and FCS were from Labtech International. Falcon microtest tissue culture plates were from Fahrenheit Lab, Milton Keynes, UK. Staphylococcal Enterotoxin B (SEB) was from Sigma.

**IFNγ and IL-4 Secretion Assays**
DPBS was purchased from Cambrex Bio Science, EDTA was from Sigma and FCS was from Labtech International. MACS Secretion Assay Kits for Human Cells were purchased from Miltenyi Biotec Ltd, Surrey, UK. Anti-CD4 Fluorescein Isothiocyanate (FITC) was purchased from BD Biosciences.

**Flow Cytometry**
Isoton II azide-free balanced electrolyte solution and flow check fluorospheres were from Beckman Coulter (UK) Ltd, High Wycombe, UK. Polypropylene tubes (5ml, 75mm×12mm) were from Starstedt Ltd, Leicester, UK.

**Annexin V staining with propidium iodide**
Hanks’ balanced salts solution (HBSS) and propidium iodide were from Sigma, and annexin V was from Alexis Corporation (UK) Ltd, Nottingham, UK.
2.2 Equipment

*Production of Ro60 MBP fusion protein*

E. coli cultures were grown in an orbital shaking incubator purchased from Infors UK, Surrey, UK. DNA was quantified and purity assessed using a Perkin Elmer Lambda 2 UV/VIS Spectrophotometer with Perkin Elmer Computerised Spectroscopy Software, version 4.3 from Perkin Elmer, Beaconsfield, UK. Agarose gels were cast in a gel tank produced by Leicester University Workshop, UK connected to a Powerpac (model 300) from Bio-Rad. PCR was performed in a Techne Unit Progene Thermal Cycler, Techne Ltd, Cambridge, UK. Images of agarose gels under UV light were captured using a UVP gel documentation system, Ultraviolet Products Ltd, Cambridge, UK.

*Purification of Ro60 (without MBP fusion)*

Bacterial and protein pellets were produced using a Beckman J2-MC with JA20 and JA14 rotors and Beckman Optima™ TLX ultracentrifuge with a TLA120.2 rotor (Beckman Coulter). Cells were lysed using a Sanyo Soniprep 150 probe sonicator from Sanyo Gallenkamp, Louborough, UK. Protein quantitation was made using an Anthos plate reader, Salzburg, Austria fitted with 492nm and 620nm filters. All SDS-PAGE and Western Blotting apparatus as well as Powerpac (model 300) were from BioRad. Western blotting Hyperfilm ECL was developed on a Compact X4 Automatic X-ray Film Processor from X-ograph Imaging Systems, Malmesbury, UK. The films were analysed using a β-Imaging Computing Densitometer with Image Quant Software version 3.3 from Molecular Dynamics, Little Chalfont, UK.

*UV and ROS induced damage of Ro60*

UVB irradiations were performed using a UVM-57 Chromato Vue lamp and WG305 filter were from Knight Optical Technologies, Surrey, UK. The amount of irradiation in mW/cm² reaching the sample was measured using an optical radiometer also from Knight Optical Technologies. Dot blotting apparatus was from BioRad connected to a vacuum pump (Warton-Marlow, Cornwall, UK).

*MCO treatment of cell lysates*

All cell manipulations were carried out in a class II safety cabinet (Walker Safety Cabinets, Derbyshire, UK) and cells were maintained in a 37°C incubator in the presence of 5% CO₂ (Sanyo).
**Cell Activation and Cell Death of PBMCs treated with Ro60**

Flow Cytometry was carried out using a Coulter Epics XL-MCL Flow Cytometer connected to EXPO 32 Acquisition and Analysis Software, both from Beckman Coulter (UK) Ltd, High Wycombe, UK.

### 2.3 Methods

#### 2.3.1 Production of a Ro60 MBP fusion protein

Recombinant Ro60 was used in all experiments and initial work attempted to subclone Ro60 into a more suitable expression vector. The cDNA encoding Ro60 was available in the expression vector pET8c for the production of recombinant Ro60 protein. The protein produced was insoluble and furthermore not expressed with a fusion tag, making it difficult to purify. Ro60 was subcloned into the expression vector pMAL for the production of a maltose binding protein (MBP) fusion protein. MBP has been shown to be effective at promoting the solubility of proteins to which it is fused (Kapust and Waugh, 1999). The aim was produce a soluble fusion protein that can be purified by affinity chromatography using MBP's affinity for maltose. This molecular project required the use of sterile media, solutions and glassware; this was kindly provided by the core technical staff in the CMHT/Department of Pathology.

#### 2.3.2 E.coli culture

LB broth was prepared according to the following recipe 10g tryptone, 5g yeast extract and 5g of sodium chloride made up to 1L and for LB agar 3.75g of agar was made up to 250ml with LB broth. Sterilisation was performed by autoclaving. To prepare liquid cultures, 5ml of sterile Luria-Bertani (LB) broth supplemented with 30μg/ml chloramphenicol and 100μg/ml of ampicillin for *E.coli* BL21(DE3)pLysS+pET8c/Ro60 culture, and 100μg/ml of ampicillin for *E.coli* TB1+pMAL were inoculated using a sterile loop with a small amount from the glycerol stock. Cultures were grown in an orbital shaker at 250rpm at 37°C overnight. Isolated colonies of the four strains were also maintained on LB agar plates. Sterile molten agar (40ml) was supplemented with appropriate antibiotics and poured into petri dishes. When set, 100μl of LB culture was spread on to the plate. Plates were incubated overnight at 37°C and those producing isolated colonies were stored at 4°C. New glycerol stocks were prepared by resuspending cells from an LB broth culture (OD$_{600}$ of 0.6) in LB with 15% (v/v) sterile glycerol and frozen in aliquots at −80°C.
2.3.3 Plasmid preparation

Plasmid purification was by a lithium chloride method to produce a high yield of high purity DNA. 100ml of overnight culture was centrifuged at 750×g for 10 minutes at 4°C and the cells were resuspended in 5 ml of Tris-EDTA (25mM and 10mM respectively), pH8 containing 50mM glucose (solution 1) and incubated for 5 minutes on ice. With gentle mixing 10ml of 0.2M sodium hydroxide containing 1% (w/v) SDS (solution 2) was added followed by incubation at room temperature for 5 minutes. 5ml of solution 3 [60ml 5M potassium acetate containing 11.5ml glacial acetic acid made up to 100ml (The resulting solution is 3M with respect to potassium and 5M with respect to acetate)] was added with vigorous mixing and incubation for 5 minutes on ice. The sample was centrifuged as above and the cell supernatant was precipitated with an equal volume of isopropanol at −20°C for 20 minutes. The precipitate was centrifuged and the pellet dissolved in 1.5ml TE (10mM Tris-HCl, 1mM EDTA, pH8). 2ml of 5M lithium chloride was added and incubated for 5 minutes on ice. The precipitate was centrifuged and supernatant was precipitated with 2 volumes of absolute ethanol at −20°C. The precipitate was centrifuged and the pellet was washed with 95% ethanol. The pellet was dissolved in 0.6ml TE with 40μg/ml DNase free ribonuclease A (RNase A), incubated at 37°C for 15 minutes and precipitated with 0.3ml 20% (v/v) polyethylene glycol (PEG) and 2.5M sodium chloride. The sample was centrifuged and resuspend in 0.5ml TE. The sample was extracted with an equal volume of phenol:chloroform (29:1) and the upper aqueous layer was retained. The extraction was then repeated with chloroform only. The sample was precipitated with 2.5 volumes of absolute ethanol and 1/10th volume of 3M sodium acetate, pH 5.2 at −20°C for 30 minutes, the sample was centrifuged at 10,000×g for 15 minutes at 4°C and the pellet was washed with 70% ethanol. The pellet was air dried and resuspended in 100μl of TE. Plasmid DNA concentration was estimated using absorbance measured at 260nm, where one absorbance unit at 260nm of double-stranded DNA equals 50μg/ml.

2.3.4 Agarose Gel Electrophoresis

The plasmid was separated by electrophoresis on a 0.7% (w/v) agarose gel to confirm the preparation was successful. To prepare the gel, agarose was heated in a microwave oven in Tris borate-EDTA buffer [TBE: 89mM Tris-borate (pH8.3) and 2mM EDTA]. 25nM ethidium bromide was added to the dissolved gel and the gel was poured into a gel cast tank and allowed to set for 1hr at room temperature (RT). Samples were diluted with gel loading solution at a ratio of 5:1 respectively and 10μl was loaded onto the gel and a 500bp ladder was also loaded. Electrophoresis was performed at 70V for 90min in TBE buffer containing 50nM ethidium bromide. The bands were visualised under UV light and images captured using a UVP gel documentation system.
2.3.5 Restriction digestion
Ro60 cDNA was excised from pET8c vector by NcoI/BamHI restriction digestion and used as a template for PCR amplification. The digestion reaction contained 5U of NcoI and BamHI, 2μg of plasmid DNA and 1x BamHI digest buffer in a final volume of 50μl and was incubated for 2 hours at 37°C. The digested products were electrophoresed on a 0.7% (w/v) agarose gel in TBE, pH8.3 and DNA of size 1.6kb was identified as Ro60 and eluted with a gel extraction kit according to the manufacturer's instructions. Briefly, under UV light the 1.6kb fragment was excised from the gel using a clean scalpel and transferred to a microcentrifuge tube. The gel slice was dissolved in Nucleo Trap buffer 1 (600μl per 100mg of agarose) and then mixed with the Nucleo Trap suspension beads (4μl per 1μg of DNA). The sample was then incubated at 50°C for 10 minutes, vortexing briefly during incubation. Subsequently the sample was centrifuged at 10000×g for 30 seconds at room temperature (RT) and the pellet resuspended in 500μl of Nucleo Trap buffer 2. After a second centrifugation the pellet was air dried for 15 minutes at RT and then resuspended in 50μl TE buffer. The sample was incubated at 50°C for 5 minutes and then centrifuged at 10000×g for 30 seconds; the supernatant containing the purified DNA fragment was then transferred to a clean microcentrifuge tube.

2.3.6 PCR Amplification
In addition to amplification of Ro60 DNA, PCR primers were synthesised with EcoRl and XbaI restriction sites on the 5’ and 3’ ends respectively, which when used for PCR would facilitate cloning of Ro60 DNA insert into pMAL vector. A PCR reaction using Pfu polymerase was set up using conditions recommended by the manufacturer. The reaction mixture contained 1x Pfu polymerase buffer containing 100mM Tris-HCl, 20mM magnesium chloride, 100mM potassium chloride, 60mM ammonium sulphate, 1% Triton X-100 and 100μg/ml BSA, pH8, 0.2mM each dNTP, 200ng template DNA, 0.5μM each primer, 2.5mM MgCl₂ and 2.5U Pfu polymerase. Temperature cycling was performed using a Techne thermocycler as follows: 1 cycle, 95°C for 45 seconds (template denaturation); 30 cycles of 95°C for 45 seconds, 50.6°C for 45 seconds (primer annealing) and 72°C for 3.3 minutes (primer extension) followed by 1 cycle, 72°C for 10 minutes.

2.3.7 Ligation of PCR insert and pMAL vector
For ligation of the Ro60 insert into the pMAL vector both vector and insert were digested with EcoRl/XbaI. The digest reactions contained 30μg of a PCR product or pMAL vector (purified by phenol:chloroform extraction followed by ethanol precipitation), 40U of each EcoRl and XbaI; and
1x EcoRI buffer in a final reaction volume of 100μl. The reaction was incubated for 2 hours at 37°C. Three ligation reactions were prepared using purified digested vector and insert. They all contained 100ng of vector and varying amounts of insert in 1:1, 3:1 and 5:1 insert:vector ratios, 1U T4 DNA ligase and 1x T4 DNA ligase buffer in a 10μl reaction buffer. The reaction was incubated at 4°C for 4 days.

2.3.8 Competent cells
E.coli TB1 and PR745 competent cells were prepared for transformation with recombinant plasmid. 100μl of an overnight culture were added to 10ml LB broth and grown to an OD<sub>600</sub> of ~ 0.5. The culture was then transferred to a flask containing 50ml LB and grown to an OD<sub>600</sub> of ~ 0.5. 100ml of LB were then added and grown to an OD<sub>600</sub> of 0.6. After cooling on ice the culture was centrifuged at 750xg for 10 minutes at 4°C. The cells were resuspended gently in 35ml of ice-cold buffer containing 30mM potassium acetate, 50mM manganese chloride, 100mM potassium chloride, 10mM calcium chloride and 15% (v/v) glycerol. The cells were centrifuged and resuspended in 8ml ice-cold buffer containing 100mM MOPS, 75mM calcium chloride, 10mM potassium chloride and 15% (v/v) glycerol. Aliquots of the cells were frozen in liquid nitrogen and stored at –80°C until required.

2.3.9 Bacterial Transformations
10μl from each ligation reaction were added to 100μl of competent cells that had been thawed on ice. 10μl of vector DNA and 10μl of UP water were used as controls. The cells were incubated on ice for 1 hour and heat shocked for 2 minutes at 42°C. They were added to 400μl of pre-warmed LB broth and incubated at 37°C in an orbital shaker at 250rpm for 1 hour. From each tube 200μl were spread onto LB agar plates supplemented 100μg/ml ampicillin and incubated at 37°C overnight.

2.3.10 Plasmid DNA mini-preparations
From the transformation plates six random colonies were selected and cultured overnight in 5ml LB with 100μg/ml ampicillin. 50μl of this overnight culture were used as a starter culture to prepare glycerol stocks from each colony. The remaining culture was centrifuged at 750g for 10 minutes at 4°C and the cell pellet was resuspended in 200μl of solution 1 (see plasmid preparation) with 100μg/ml RNase A. 200μl of solution 2 (see plasmid preparation) was added with gentle mixing. 100μl of ice-cold solution 3 (see plasmid preparation) was added and the tube inverted several times. The solution was microfuged for 5 minutes at room temperature. The supernatant was transferred to a clean tube and was extracted with phenol:chloroform (29:1) and precipitated with ethanol, after
which the pellet was resuspended in 50μl of TE with 20μg/ml RNase A. An EcoR1/Xba1 restriction digestion of pMAL was used to identify which transformed colonies contained the Ro60 insert. The digestion reaction contained 10μl of plasmid DNA mini-preparation, 20U EcoR1 and 20U Xba1, Xba1 digest buffer containing 50mM sodium chloride, 10mM Tris-HCl, 10mM magnesium chloride, 1mM DTT and 100μg/ml BSA, pH 7.9 in a final volume of 20μl. The reaction was incubated for 2 hours at 37°C. The digested products were electrophoresed on a 0.7% (w/v) agarose gel and a band of 1.6kb was identified as the Ro60 insert.

2.3.11 Trial Overexpression
Overexpression experiments were carried out by adding 1ml of overnight culture of E.coli TB1+pMAL/MBP-Ro60 to 20ml of LB supplemented with 100μg/ml ampicillin and 0.2% (w/v) glucose. Cells were grown at 37°C in a shaker at 250rpm for 1 hour. 0.5ml of uninduced cells was removed and stored for analysis, and the culture was induced by addition of 0.1mM IPTG. Cells were grown for 3 hours and 0.5ml of induced cells was removed for analysis. Uninduced cells were centrifuged at 750g for 10 minutes at 4°C and resuspended in 100μl of TE. Induced cells were centrifuged and resuspended in appropriate volume based on comparison of uninduced and induced sample absorbance at 600nm, so that each sample contained equal amounts of cells. The samples were stored at ~20°C for SDS-PAGE analysis.

2.3.12 SDS-PAGE
SDS-PAGE was carried out using a mini-Protean II gel electrophoresis system (BIO-RAD, Hertfordshire, UK). 10% w/v acrylamide:bisacrylamide (3% (w/v) bisacrylamide) mini-gels (8x10x0.07cm) containing 375mM Tris-HCl, pH gel buffer and 0.1% SDS (w/v) were polymerised with 100μl 10% (w/v) ammonium persulphate and 4μl TEMED in a final volume of 10ml. The gels were overlayed with a 5% (w/v) acrylamide:bisacrylamide (3% (w/v) bisacrylamide) stacking gel containing 125mM Tris-HCl, pH6.8 gel buffer and 0.1% (w/v) SDS. Samples were diluted 1 volume of sample to 0.5 volume of loading buffer (62.5mM Tris-HCl, pH6.8, 25% (v/v) glycerol, 0.5% (w/v) SDS, 0.01% (w/v) bromophenol blue, 100mM DTT) and heated for 5 minutes at 95°C prior to loading. Precision, broad range prestained protein standards (Bio-Rad) were used as molecular weight markers. Samples were electrophoresed with 25mM Tris base, 192mM glycine and 0.1% (w/v) SDS running buffer, at 200V (constant voltage) for 40 minutes.
2.3.13 *Coomassie blue staining*

Gels were stained with Coomassie brilliant blue R250 0.5% (w/v) in methanol:ultrapure (UP) water:glacial acetic acid; 5:4:1 for 1 hr and destained with methanol:UP water:glacial acetic acid; 7:2:1 for 2 hr with four changes of the destaining.

2.3.14 *Cell lysis*

Cells from overexpression of Ro60 in *E.coli* TB1+pMAL/MBP-Ro60 were lysed and analysed by SDS-PAGE to see if overexpressed Ro60 was present in the supernatant or insoluble fraction. To be defined as a soluble protein, it should be retained in the supernatant after centrifugation at 100,000×g for 45 minutes (Arakawa and Timasheff, 1985). Overexpressed cells were harvested by centrifugation at 750×g for 10 minutes at 4°C. The pellet was resuspended in 0.25 volumes of lysis buffer (20mM Tris-HCl, pH 8, 200mM NaCl, 1mM EDTA, 1mM PMSF) and frozen at −20°C. The sample was thawed in cold water and sonicated 7 times for 10 seconds using a Sanyo Soniprep 150 probe sonicator and the protein released was monitored using the Bio-Rad protein assay. 100μl of lysed cells were collected for SDS-PAGE and the remaining lysate was centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant was removed and the insoluble fraction resuspended in 0.25 culture volumes of lysis buffer. 1ml of supernatant was centrifuged at 100,000×g for 45 minutes. All fractions were subsequently analysed by SDS-PAGE.

2.3.15 *Affinity purification*

500μl of amylose resin was centrifuged at 9000×g for 1 minute and the supernatant removed, to wash the resin, it was resuspended in 1.5ml of lysis buffer (section 2.3.13) and centrifuged at 9000×g for 1 minute and this process was repeated and after the second wash the resin was resuspended in 500μl of lysis buffer. The resin was then added to 5ml of cell lysis supernatant from overexpressed cells and incubated on ice for 15 minutes. The resin was then centrifuged at 750×g for 5 minutes and the supernatant was removed and stored at 4°C. The resin was the washed twice with lysis buffer and resuspended in 500μl. 100μl were kept for analysis and remaining resin was incubated on ice for 15 minutes with 5ml lysis buffer containing 10mM maltose to elute any bound protein. The resin was centrifuged at 750×g for 5 minutes, the supernatant was removed and the resin was resuspended in 500μl lysis buffer without maltose. The eluted sample was concentrated to 500μl in Centricon centrifugal filter devices from Amnicon.
2.3.16 *Factor Xa cleavage*

The 500μl of Ro60/Mal fusion eluted from the amylose beads was further concentrated to at least 1mg/ml (Microcon centrifugal filter devices from Amicon) for cleavage with factor Xa. A reaction was set up using 10μg of protein in a final volume of 20μl with 2% (w/v) Factor Xa. A control reaction was set up containing 20μl of fusion protein only. The tubes were incubated for 4 hours at room temperature and samples were then analysed by SDS-PAGE.

2.3.17 *Isolation of inclusion bodies*

In most cases overexpressed protein in *E. coli* accumulates intracellularly in an insoluble form resulting in cytoplasmic granules called inclusion bodies. A method to isolate inclusion bodies using lysozyme/DNAase/detergent to lyse cells and detergent to wash inclusion bodies was used (Marston, 1987). Samples were taken during the procedure for SDS-PAGE as the inclusion bodies may have been solubilised by detergents used during cell lysis and pellet wash steps. Overexpressed cells were resuspended in 0.25 culture volumes of lysis buffer (section 2.3.13) and incubated at room temperature for 20 minutes with 150μg/ml lysozyme. 0.15% (v/v) Nonidet P-40 detergent was added and the cell lysate incubated at 37°C until viscous. 12.5μg/ml deoxyribonuclease 1 (DNAse1) was added and the lysate was incubated for 30 minutes or until no longer viscous. The lysate was centrifuged at 9000×g for 15 minutes at 4°C and the supernatant was removed and kept for analysis. The cell lysis pellet was washed by incubating with 10ml of lysis buffer (section 2.3.13) containing 0.5% (v/v) Triton X-100. The sample was centrifuged at 9000×g for 15 minutes at 4°C and the wash buffer transferred to a clean tube. The pellet was resuspended in 10μl of 8M urea.

2.3.18 *Purification of Ro60 (without MBP fusion)*

It was decided to attempt to overexpress and purify Ro60 using the original pET8c vector. Overexpression of Ro60 in the original pET8c vector was known to produce Ro60 in inclusion bodies and the isolation of these was a relatively simple procedure that resulted in Ro60 of sufficient purity (Peek *et al.*, 1994). The main problem was that to obtain Ro60 from the inclusion bodies then had to be solubilised with agents that resulted in protein denaturation. To obtain a soluble protein, renaturation of the denatured protein had to be encouraged by removal of the denaturant.

In a sterile flask 700μl of overnight culture of *E.coli* BL21 (DE3) pLysS+pET8c/Ro60 were added to 70ml of LB supplemented with 100μg/ml ampicillin and 30μg/ml chloramphenicol. Cells were
grown for 3 hours at 37°C and then the culture was induced by addition of 0.1mM IPTG. Cells were grown for 3 hours at 37°C and then harvested by centrifugation at 750×g for 10 minutes at 4°C and resuspended in 0.25 culture volumes of 20mM Tris, pH7.4 containing 200mM NaCl and 1mM EDTA and stored at −20°C overnight. Cells were lysed by freeze/thawing 3 times with dry ice and incubated with 5μg/ml DNAase for 20 minutes at 37°C. Lysozyme was added at a concentration of 10μg/ml and the lysate was incubated for 30 minutes on ice. The lysate was incubated on ice for 15 minutes with 0.2% (v/v) NP-40 and 10mM EDTA and then sonicated 18 times for 10 second pulses on ice after the addition of 0.2mg/ml zwittergent 3-14. The lysate was layered onto 1ml of 40% (w/v) sucrose in 8 Oakridge centrifuge tubes. These were spun at 10,000×g for 45 minutes. Each pellet was resuspended in 2.5ml of 6M guanidine-HCl prepared in lysis buffer (section 2.3.13). The samples were pooled (approximately 22ml) and then diluted 20 times with PBS, pH 8.

2.3.19 Silver Staining of SDS-PAGE gels
Purity of isolated Ro60 was assessed by SDS-PAGE visualised with silver staining. Gels were fixed with methanol:UP water:glacial acetic acid; 45:45:10 for 25 minutes and then in methanol: UP water: glacial acetic acid; 5:92.5:2.5 for 20 minutes. The gels were then washed for 90 minutes in UP water. They were then stained with 0.1% w/v silver nitrate for 20 minutes, rinsed with UP water and developed with 400mM sodium carbonate containing 0.064% (v/v) formaldehyde. When bands were developed the reaction was stopped with 1% (v/v) glacial acetic acid.

2.3.20 Western Blot Analysis
It was confirmed that the isolated protein was Ro60 by Western blotting with anti-Ro60 antibodies. Approximately 5μg of Ro60 protein separated by SDS-PAGE was transferred to PVDF membrane for Western blot analysis using a mini trans-blot electrophoretic transfer cell. A gel sandwich for transfer to PDVF membrane was set up according to the manufacturer’s instructions. The gel sandwich was placed in the cassette and the transfer was electrophoresed for 1 hr at 63V using 37mM Tris base, 357mM glycine, 15% v/v methanol transfer buffer. After transfer the membrane was rinsed with Tris buffered saline (TBS) (40mM Tris-HCl, pH7.6, 275mM NaCl) and membrane was blocked with TBS with 0.2% Tween 20 (TBS/T) containing 5% (w/v) dried skimmed milk (TBS/T) for 1 hour at room temperature. The membrane was washed with TBS/T and incubated with 50μl anti-Ro2 at 1:500 dilution with TBS/T for 1 hour. The membrane was washed with TBS/T and incubated with HRP-labelled anti-human IgG at a 1:10,000 dilution with TBS/T for 1 hour. The membrane was washed with TBS/T and HRP label detected by chemiluminescence using ECL Western blotting detection reagents. Equal volumes of reagents 1 and 2 were mixed and applied to
the membrane with incubation for 1 minute. Excess reagent was removed and the membrane sealed in a plastic wallet. The wallet was placed in a hyper film cassette and exposed in the dark room to high performance chemiluminescence film for 30 seconds. The film was then developed in a film processor.

2.3.21 Concentration of Ro60
The diluted protein sample was added to dialysis tubing with a flat width of 76mm, an average diameter of 49mm and a molecular weight cut off of 12,000 Da. The tubing was prepared by washing in running water for 3-4 hours to remove glycerin. To remove sulphur compounds it was treated with 0.3%(w/v) sodium sulfite solution at 80°C for one minute, washed with hot water (60°C) for 2 minutes, followed by acidification with 0.2% sulphuric acid and rinsed with hot water to remove the acid. The tubing was then stored in sterile PBS at 4°C. The diluted protein (typically 450ml from section 2.3.17) in dialysis tubing was covered with PEG of molecular weight 8000Da and left overnight at 4°C to concentrate. The concentrated protein was moved from the dialysis tubing. This was dialysed overnight against PBS to remove residual guanidine-HCl. The protein was then aliquoted and stored at -80°C.

2.3.22 Protein Assays
Initially protein determination was made using the BioRad protein assay reagent based on the Bradford assay (Bradford, 1976). A 0.5mg/ml BSA standard was prepared in UP water and stored in aliquots at -20°C. This was used to prepare six standards in triplicate of 0-0.5mg/ml in a final volume of 10μl in a 96 well micro-titre plate. Sample (10μl) in triplicate was placed into the 96-well plate. Samples with a protein concentration greater than 0.5mg/ml were diluted prior to measurement so that the sample absorbance was within the range of the standard curve. 200μl BioRad protein dye solution, pre-diluted 1 in 5, was added to each well. Plates were incubated at RT for 10 minutes before being read spectrophotometrically at 620nm. For measurement of protein of concentrations of less than 100μg/ml the micro BCA protein assay was used (Smith et al, 1985). A 2mg/ml BSA standard was diluted to give nine standards in triplicate 0-200μg/ml in a final volume of 8ml. 150μl of each standard and 150μl of sample was placed into a 96-well plate. 150ml of working reagent containing 25ml of BCA reagent A, 24 ml of BCA reagent B and 1ml of BCA reagent C was added into each well was incubated for 2hr at 37°C before being read spectrophotometrically at 562nm.
2.4 UV and ROS induced damage of Ro60

The purified protein was used to investigate the effect of UVB irradiation on Ro60 using a range of doses. The effect of ROS exposure was also investigated as an indirect effect of UVB by using the metal catalyzed oxidation system (MCO), H$_2$O$_2$/Cu, using a range of H$_2$O$_2$ concentrations. The MCO system is known to generate OH$^\cdot$ by a Fenton reaction and was chosen because OH$^\cdot$ is a major initiator of ROS damage to proteins.

2.4.1 UVB Irradiations

Ro60 in PBS was irradiated with UVB using a UVM-57 Chromato Vue lamp with a peak wavelength of 305nm. 400 or 600μl of protein solution were irradiated on ice in a 1.5ml eppendorf which had been cut down to half height. A WG305 filter which filtered out wavelengths below 305nm was placed between the sample and the lamp source; the surrounding area was covered by a protective shield. The filter was used to remove all contaminating UVC from the broad band UVB lamp. The amount of irradiation in mW/cm$^2$ reaching the sample was measured using an optical radiometer and the time required to achieve doses of up to 20 times the MED (minimal erythemal dose) was calculated, where 1MED for UVB equals approximately 40mJ/cm$^2$ (for average skin type) and represents the dose of UV required for the generation of UV-induced erythema 24 hours after irradiation. Non-irradiated controls were incubated on ice in the dark for the same periods of time. During an irradiation time course 50 or 200μl aliquots at time points were removed after moving the sample away from the lamp source and replacing afterwards in the same position. This enabled the filter to be removed to take a sample and the lamp to be left on continuously without exposing the sample to unfiltered irradiation.

2.4.2 MCO Treatment of Ro60

In an attempt to induce oxidative damage to Ro60 the H$_2$O$_2$/Cu MCO system was used. Ro60 was incubated with increasing concentrations of H$_2$O$_2$ at a constant Cu concentration. A solution of H$_2$O$_2$ 10 times concentrated in PBS was prepared using a H$_2$O$_2$ stock of 10M. This was then serially diluted to provide a range of treatment concentrations from 25-400μM. For treatment of Cu at 20μM a solution of 10 times concentrate in PBS was prepared. H$_2$O$_2$ and Cu were then diluted 10 times with Ro60 in a final volume if 200μl. The samples were incubated for 1 hr at 37°C and the reaction stopped by incubation on ice prior to Western blotting and dot blot analysis.
In subsequent experiments antioxidants were used to investigate the mechanisms of Ro60 damage. Ro60 protein samples of 50μl were incubated for 15 minutes at RT with final concentrations of 200mM Mannitol (Kang et al, 2001), 20μM DFO (Hermes-Lima et al, 2001) 50mM DMSO and 250μM (Akagawa and Suyama et al, 2002) using 10 times concentrate stocks. Subsequently they were treated with hydrogen peroxide and copper and analysed for protein carbonyls using Western Blotting.

2.4.3 Dot Blotting and Western blotting

Ro60 treated with UV-B and MCO was analysed by Western blotting and dot blotting with anti-Ro antibodies. For the latter, Ro60 was applied directly to the membrane and reflected the native state of the purified protein. This may generate different results to Western blotting where samples were denatured during SDS-PAGE electrophoresis. For the dot blot assay the plastic components of the apparatus were soaked overnight in detergent then rinsed well before each use to prevent sample cross contamination. 3mm filter paper and nitrocellulose membrane were cut to cover the 96 wells on the dot blot apparatus and were pre-soaked in PBS. These were then layered onto the rubber seal inside the dot blot apparatus, the top placed on and the screws fastened evenly to finger tightness. 50μl of PBS was then applied to each well ensuring no bubbles were present, 25μl of sample was then applied to appropriate wells. A vacuum was created using a vacuum pump and this was then connected to the dot blot apparatus just long enough for the samples to be drawn onto the membrane and then the tube disconnected. The membrane was then removed and immunostained as was described for the Western blotting procedure (section 2.3.19).

Protein carbonyl dot blot assay

An attempt was made to establish a dot blot assay based on the method of Robinson et al, 1999 to measure protein carbonyl groups that have been shown previously to be a marker of protein oxidative damage. Carbonyl groups were first derivatised with DNPH and then detected using a polyclonal anti-DNP-KLH rabbit IgG antibody.

10μl PBS was added to sample tubes containing 25μl samples of Ro60 irradiated with UVB or exposed to MCO. To this 25μl of 100μg/ml DNPH in 2M HCl was added (Keller et al, 1993). The tubes were incubated for 5 minutes at RT and then 25μl was applied to the dot blotter in duplicate. Samples were drawn onto the membrane by vacuum. Controls were included in each set of samples, these were underivatised Ro60 and PBS incubated with DNPH. The membrane was then removed and washed for 30 minutes in 2M HCl followed by 3 times 10 minute washes in PBS to remove any
unbound DNPH. The membrane was blocked with TBS/T containing 5% (w/v) dried skimmed milk (TBS/T) for 1 hour at room temperature and then incubated with anti-DNP at a 1:5000 dilution in blocking reagent for 1 hour at room temperature. The membrane was washed with TBS/T and incubated with anti-rabbit IgG-HRP conjugate at a 1:25,000 dilution in blocking reagent for 1 hour at room temperature. Detection was performed as for Western Blotting (section 2.3.19).

**Protein Carbonyl Western blot Assay**

Due to technical difficulties with the dot blot technique for measurement of protein carbonyls, a Western blotting method was also developed (Levine *et al.*, 1994). When using the dot blot technique problems were frequently encountered with free DNPH binding to the nitrocellulose membrane leading to a false positive result in the sample blank. This did not occur with the Western blotting procedure as free DNPH was separated from protein bound DNPH during SDS-PAGE electrophoresis. Derivitisation and immunostaining were performed as described for the dot blotting method. Prior to SDS-PAGE electrophoresis samples were neutralised with 2M Tris/30% glycerol. SDS-PAGE and Western blot analysis were carried out essentially as described in sections 2.3.11 and 2.3.19.

**2.5 MCO treatment of cell lysates**

Cultured human normal primary dermal fibroblast cells were used as a source of cell lysate to investigate whether changes induced by MCO on purified Ro60 could be reproduced with Ro60 present within a cellular environment.

**2.5.1 Maintenance of NDF cell lines**

The cells were maintained Dulbecco’s Modified Eagles Medium supplemented with 10% Foetal Calf Serum and 5μg/ml Gentomycin in an 80cm² cell culture flask at 37°C in the presence of 5% (v/v) CO₂. The cells were seeded at 2×10⁶ per ml. The cell medium was changed weekly and once a week, when confluent, the cells were subcultured. Cells were not used after passage number 20. To subculture, the medium was removed and the cells were washed twice with 10ml Dulbecco’s Phosphate Buffered Saline and once with 2.5ml of trypsin-EDTA solution (0.05%(w/v) trypsin, 0.02% (w/v) EDTA and 145mM sodium chloride (TE)). Cells were detached from the flask by incubation with 2.5ml of TE at RT, agitating the flask until all the cells were seen under the microscope to be detached (approximately 3 minutes). The TE was then neutralised by the addition of 10ml of medium and the cells were split and centrifuged at 250×g for 5 minutes. Half the cells
were resuspended in 5ml medium and transferred to a new flask containing 25ml of fresh medium. The other half was used to prepare cell lysates.

2.5.2 Preparation of cell lysate
The cell pellet, prepared as described in the previous section, was washed twice with 10ml DPBS and then resuspended in 75µl of lysis buffer (50mM Tris-HCl containing 3mM EDTA, 5mM magnesium acetate, 50mM potassium chloride, 3mM β-mercaptoethanol, 5mg/ml Leupeptin, 5µg/ml Antipain, 5µg/ml Pepstatin A and 5µg/ml Chymostatin) on ice and stored at -80°C as previously described by Yamaguchi et al, 1996. Cells lysates were thawed and homogenised 15 times using a sterile eppendorf hand homogeniser on ice. The homogenate was centrifuged at 10000g for 5 minutes at 4°C and the supernatant collected. The protein concentration of the supernatant was determined using the BioRad protein assay using the standard assay in micro-plate format as described in the manufacturers’ instructions (see section 2.3.21).

2.5.3 Treatment of cell lysate with hydrogen peroxide and copper
The cell lysate was diluted with PBS to a protein concentration of 1.5mg/ml, solutions H₂O₂ and Cu²⁺ 10 times concentrate were prepared and serial diluted to provide final treatment concentrations of 750 and 1200µM H₂O₂ and 300µM Cu²⁺. (Concentrations were equivalent to those used for treating Ro60 when based on the total protein concentration). H₂O₂ and Cu²⁺ were then diluted 10 fold with cell lysate in a final volume of 50µl and incubated at 37°C for 1 hour. The reaction was stopped by incubation on ice.

Measurement of Ro60 and protein carbonyls in treated cell lysates
From the treated sample 7.5µg of protein was loaded on a gel for Western blot analysis with anti-Ro60 serum as described in section 2.3.19. The remainder of the sample was derivatised with DNPH and analysed for protein carbonyls by Western blotting as described in section 2.4.3. The final amount of protein loaded on the gel was 10µg.

2.6 Cell Activation and Cell Death of human PBMCs treated with Ro60
After treatment with Ro60 and ROS damaged Ro60, IFN-γ-secreting and IL-4-secreting cells were analysed by flow cytometry as a measure of cell activation. Cell death response was also measured by annexin V staining with propidium iodide for the detection of viable, apoptotic and dead cells also using flow cytometry.
2.6.1 Isolation of human peripheral blood mononuclear cells

20ml of venous blood was drawn from the antecubital vein of human volunteers and mixed with lithium heparin anticoagulant (the study was approved by the Leicestershire Health Authority Research Ethics Committee and informed written consent was obtained before the collection of blood samples). Peripheral blood mononuclear cells were isolated from the blood by Histopaque gradient separation, within two hours of venepuncture. The blood was diluted with an equal volume of PBS containing 2 mM EDTA (PBS/EDTA) and layered over a total volume of 20ml of Histopaque-1077, at room temperature, divided into four 50ml centrifuge tubes. For blood separation the tubes were centrifuged in a swinging-bucket rotor, without brake, at 400×g for 30 minutes at room temperature. After centrifugation the upper layer containing diluted autologous plasma collected and kept for use as a culture medium supplement. The opaque interface containing PBMCs was then removed with a plastic pasteur pipette to a clean tube. The cells were washed by dilution with 40ml PBS/EDTA and centrifugation at 300×g for 15 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 10ml PBS/EDTA and centrifuged at 200×g for 5 minutes at room temperature. The cells were then resuspended in 2ml PBS/EDTA containing 10% autologous plasma and stored overnight at 4°C. A haemocytometer counting chamber was used to determine cell number and viability was assessed by staining dead cells with 0.4% w/v Trypan Blue solution: cell solution (20μl) was mixed with 20μl Trypan Blue solution and 10μl was applied to a counting chamber. Live cells (translucent) and dead cells (blue) were counted in 5 of 25 squares within the triple-line markings on the chamber. The number of cells per ml of suspension was calculated by multiplying the number of cells in 25 squares by the dilution factor and by a factor of 10^4. Viability was assessed as the percentage of live cells.

2.6.2 Treatment of PBMC with antigen

PBMCs were centrifuged at 200×g for 5 minutes at room temperature and resuspended at a cell concentration of 0.25×10^6 cells/ml RPMI 1640 containing 10% FCS. Antigens SEB, Ro60 and ROS damaged Ro60, which had been filter sterilised and stored in aliquots at -80°C, were incubated with 150μl of cell suspension at a final concentration of 10μg/ml in 96-well plastic cell culture plates for 6hr at 37°C in the presence of 5% (v/v) CO_2. Cells with no antigen were included as controls. After treatment the number of viable IFN-γ-secreting and IL-4-secreting cells was analysed by flow cytometry as a measure of cell activation. Cell death response to antigen was also measured by annexin V staining with propidium iodide for the detection of viable, apoptotic and dead cells also using flow cytometry.
2.6.3 *IFN* γ and *IL-4* Secretion Assays

Two secretion assays were performed i) for measurement of CD4 positive IFN γ secreting cells using an IFN γ catch antibody with the corresponding phycoerythrin (PE) detection antibody and a CD4 fluorescein isothiocyanate (FITC) detection antibody and ii) IFN γ and IL-4 secreting cells using IFN γ catch antibody with FITC detection antibody and IL-4 catch antibody with PE detection antibody; using MACs cytokine secretion assay kits and protocol for both assays was as follows: treated cells (see section 2.6.2) were transferred to 12ml centrifuge tubes, wells washed once with RPMI containing 10% FCS by centrifugation at 200×g for 5 minutes at room temperature. The cell pellets were resuspended in 80μl of RPMI containing 10% FCS. This was followed by 5 minutes incubation on ice with 5μl of single or dual catch reagents (1:18 antibody dilution) RPMI containing 10% FCS was pre-warmed to 37°C and 1ml was added to the cells and they were transferred to a 24-well plastic cell culture plate and incubated for 45 minutes at 37°C in the presence of 5% v/v CO₂. After incubation the cells were centrifuged at 200×g for 5 minutes at room temperature and resuspended in 80μl PBS containing 2mM EDTA and 1% FCS and incubated on ice for 10 minutes with 5μl of the detection reagent(s) (1:18 antibody dilution) with 5μl anti-CD4 (1:18 antibody dilution) if required. Cell were washed with 1ml of PBS containing 2mM EDTA and 1% FCS and resuspended in 0.5ml of PBS containing 2mM EDTA and 1% FCS and subsequently analysed by flow cytometry.

2.6.4 Flow Cytometry for IFN γ and IL-4 secreting cells

Using the flow cytometry data acquisition software protocols were produced for the analysis of CD4 positive IFN γ secreting cells and IFN γ and IL-4 secreting cells with the help of Caroline Woolston, Department of Pathology, University of Leicester, UK. A live lymphocyte population based on size and granularity was identified based on analysis of light scatter. This was used to set a gate for the analysis of IFN γ, IL-4 or CD4 positive cells. For a typical histogram of forward scatter and side scatter with a log scale see figure 5.2. To set up the protocol for the detection of PE and FITC fluorescence, cells with no fluorescence staining were used for calibration and cells labelled with FITC only and PE only were used to set compensation so that there was no cross-talk between the two fluorescence channels. After the acquisition of 10,000 events the data was presented as a histogram of channel 2, for PE fluorescence (excitation 488nm and emission 578nm), versus channel 1, for FITC fluorescence (excitation 488nm and emission 520nm), using a log scale, together with a calculated percentage of the PE and FITC positive cells using the EXPO 32 Acquisition and Analysis Software connected to the Coulter Epics XL MCL Flow Cytometer (Beckman Coulter (UK) Ltd). For each set of antigen treated lymphocytes a sample with no
fluorescence staining was analysed and any background fluorescence was subtracted from the values obtained for the samples.

2.6.5 Annexin V staining with propidium iodide
The effect of antigen treatment on cell death responses was also investigated. Apoptotic, necrotic and viable cells were distinguished using flow cytometry by dual staining the cells with FITC-conjugated annexin V and propidium iodide (PI) (Koopman et al, 1994). After treatment of PBMC with antigen in vitro the cells were centrifuged at 200×g for 5 minutes at room temperature and resuspended in 1ml of Hanks’ balanced salts solution (HBSS), centrifuged again at 200×g for 5 minutes at room temperature and resuspended in annexin V buffer containing 10mM HEPES, 159mM sodium chloride, 5mM potassium chloride, 1.8mM calcium chloride and 1mM magnesium chloride, pH 7.4. Cells were incubated, in the dark, for 15 minutes with a final concentration of annexin V of 2.5μg/ml. PI 1μg/ml was added prior to analysis by flow cytometry using a protocol in use by Caroline Woolston. Using a live lymphocyte gate 10,000 events were acquired and data presented as a histogram of channel 3, for PI fluorescence (excitation 488nm and emission 617nm), versus channel 1, for annexin V FITC fluorescence (excitation 488nm and emission 519nm), using a log scale. Background fluorescence of each set of lymphocyte samples was subtracted from the analysis of cells with no fluorescence staining.

2.6.7 Statistical Analysis of Data
Bands on Western blot films were quantified using a β-Imaging Computing Densitometer with Image Quant Software version 3.3. The data obtained from Western blot and flow cytometry analyses were expressed as fold change from control and statistical analyses were performed using GraphPad Instat Version 3 (GraphPad Software Inc, San Diego, CA, USA).
CHAPTER 3:

PURIFICATION OF HUMAN RECOMBINANT Ro60
3.1 Introduction

The development of bacterial systems enabling the expression of high levels of cloned genes was a major advance of genetic engineering (Marston, 1986). However, the highly expressed protein often accumulates in the form of insoluble inactive inclusion bodies and this presents a major problem for the recovery of a high yield of protein in its native conformation and also biologically active form (Schein, 1989). The advantage of the presence of inclusion bodies is that they can be isolated and general protocols have been established that have enabled isolation with relatively high purity of many recombinant proteins (Marston et al, 1984; Schoner et al, 1985). From this form it is possible to recover soluble and biologically active protein by using agents that release the protein from inclusion bodies into solution and the solubilised protein can then be refolded to attain its native conformation by gentle removal of the solubilisation agent under appropriate conditions (Marston and Hartley, 1990). There are many factors which influence the refolding process and these depend on the nature of the individual protein and thus the process has to be optimised for the refolding of a specific protein (Clarke et al, 1999).

The aim of the work in this chapter was to purify the human recombinant protein Ro60 where it was used as a model SLE autoantigen for work in subsequent chapters. In human cells this protein forms part of a Ro ribonucleoprotein complex in the cytoplasm of the cell with one of four small cytoplasmic Y RNA molecules and at least two other proteins namely Ro52 and La (Pruijn et al, 1991). The components of this complex are also autoantigens in SLE (Maddison and Reichlen, 1977) but Ro60 was chosen in preference to the others because of the association of its autoantibodies and the development of photosensitive skin lesions in SLE (Mond et al, 1989). The work with the purified protein focused on investigating ROS damage in vitro of this model autoantigen. The source of ROS capable of inducing oxidative damage in vivo may be cellular (see section 1.11) but could also involve those generated by UV in sunlight (Lawley et al, 2000) perhaps accounting for association of Ro60 autoantibodies with photosensitive skin lesions. The reason ROS modified protein was generated was because ROS have been implicated in the induction of autoimmunity via the initiation of protein oxidation reactions leading to the recognition of antigen as ‘non-self’ (see section 1.12). The native and modified protein was then used to investigate the immune response to these antigens in vitro using peripheral blood lymphocytes from SLE subjects positive for the presence of this autoantibody.

For the purification of human recombinant Ro60, the cDNA encoding Ro60 in the expression vector pET8c in E.coli was kindly given by Dr. G. Pruijn, University of Nijmegen, The Netherlands. He
advised that in contrast to La which is normally expressed in soluble form, the Ro proteins (Ro60 and Ro52) were completely insoluble (inclusion bodies) and it appeared almost impossible to develop a satisfactory renaturation procedure for these proteins (personal communication). An alternative way of improving the solubility of recombinant proteins and avoiding the 'inclusion body problem' has been shown to be the use of fusion proteins, originally employed to aid purification by use of an affinity domain (Uhlen et al., 1993). The use of several highly soluble fusion partners has been shown to improve solubility of protein, although this effect is still poorly understood (La Vallie et al., 1993; Nygren et al., 1994; Samuelsson et al., 1994).

A recent study was carried out to investigate the solubility of several fusion proteins using three soluble fusion partners each combined with one of six normally insoluble recombinant proteins (Kapust and Waugh, 1999). The aim was to see if all soluble fusion partners were equally likely to aid the solubility of their insoluble passenger proteins and also to investigate if the solubility of insoluble passenger proteins is always improved when using this technique. The results showed that the maltose binding protein was far more effective at solubilising agent than the other two fusion proteins, where the solubility of all six passenger proteins was improved by fusing them to this fusion protein. Fusion to the maltose binding protein also demonstrated that this protein could in some cases promote proper folding of the fused protein into its biologically active conformation.
3.1 AIMS

The overall aim of the work in this chapter was to purify the human recombinant protein Ro60 so that it could be used as a model SLE autoantigen in the following investigations. It was required for two sets of *in vitro* experiments, firstly to produce oxidatively modified Ro60 protein by exposure to reactive oxygen species and subsequently to observe if either form of this protein induces an autoimmune response using peripheral lymphocytes from SLE subjects positive for the presence Ro60 autoantibody. The first specific aim was to subclone Ro60 available in the expression vector pET8c for the production of insoluble recombinant Ro60 protein into expression vector pMAL for the production of a maltose binding protein fusion protein and the second aim was to assess the solubility of the overexpressed Ro60 maltose binding protein. The final aim was to purify the protein using the maltose binding protein system if it was proved to be soluble, and if not, a renaturation procedure for this protein would need to be revised to purify the protein following overexpression in the original vector.
3.3 METHODS

3.3.1 Subcloning of Ro60
Ro60 available in the expression vector pET8c was subcloned into the vector pMAL for the expression of a Ro60 maltose binding fusion protein. Ro60 cDNA was excised from the pET8c vector using methods 2.3.1-2.3.4. The DNA was then amplified by PCR (section 2.3.5). The PCR primers were synthesised with EcoRI and XbaI restriction sites on the 5' and 3' ends respectively, which when used for PCR would facilitate the cloning of the Ro60 DNA insert into the pMAL vector. The Ro60 insert was then ligated into the pMAL vector as described in section 2.3.6 and the recombinant vector was transformed into E.coli TB1 (section 2.3.7 and 2.3.8). Restriction digestion of pMAL was then used to identify which transformed colonies contained the Ro60 insert (section 2.3.7).

3.3.2 Trial overexpression of the Ro60 maltose binding protein fusion
E.coli TB1+pMAL/MBP-Ro60 cells were overexpressed (section 2.3.10) and the cells analysed by SDS-PAGE with Coomassie blue staining (sections 2.3.11-2.3.12). The cells were lysed and the solubility of the fusion protein was assessed as described in section 2.3.13. A method was used to isolate inclusion bodies from overexpressed E.coli TB1+pMAL/MBP-Ro60 (section 2.3.16).

3.3.3 Purification of the Ro60 maltose binding protein
Methods for affinity purification and cleavage by Factor Xa to release the maltose binding protein from Ro60 were tested as described in section 2.3.14-2.3.15.

3.3.4 Purification of Ro60 from E.coli BL21 (DE3) pLysS+pET8c/Ro60
Ro60 inclusion bodies were isolated from E.coli BL21 (DE3) pLysS+pET8c/Ro60 using the method described in section 2.3.17. This section also describes the conditions used for renaturation of the inclusion bodies. The purity of the isolated Ro60 was assessed by SDS-PAGE visualised with silver staining (section 2.3.18). Anti-Ro60 antibodies were used to confirm the protein was Ro60 by Western blotting as described in section 2.3.19. The purified protein was concentrated and the final concentration was determined (sections 2.3.20 and 2.3.21).
3.4 Results

3.4.1 *E. coli* BL21(DE3)pLysS+pET8c/Ro60

3.4.2 pET8c vector and excised DNA insert

Plasmid preparations of pET8c of expected size 4.6kb (G. Pruijn, University of Nijmegen, personal communication) and pMAL of expected size 6.6kb (Reed *et al*., 1993) were visualised following separation by agarose gel electrophoresis (Figure 3.1: Lanes 3 and 8; and Figure 3.2: Lane 7 respectively). Multiple bands seen for pMAL may represent different conformations, open circular, supercoiled and closed circular of the plasmid; formed during the isolation procedure that would migrate at different rates. The expected size does not correlate with the fragments separated by the DNA ladder but these are fragments of linear DNA that will migrate at a different rate. Ro60 DNA was excised from pET8c by a NcoI/BamH1 restriction digest and gave a DNA band of expected size 1.6kb (Figure 3.1: Lane 1 and 10). This fragment was gel-eluted and used as a template for PCR with Ro60 specific primers. PCR products were separated by agarose gel electrophoresis and bands of size 1.6kb are shown (Figure 3.3: Lanes 4 and 6). No template and no primer controls gave no bands (Figure 3.3: Lanes 7 and 8).

![Figure 3.1: Preparation of the Ro60 cDNA for subcloning into the pMAL vector for the expression of a Ro60 maltose binding fusion protein. pET8c vector was purified from *E. coli* BL21 (DE3) pLysS culture using the lithium chloride plasmid purification method and subsequently Ro60 cDNA was excised from pET8c vector by NcoI/BamH1 restriction digest. Electrophoresis on a 0.7% (w/v) agarose gel of the pET8c plasmid preparation and restriction digest sample confirms the plasmid isolation and excision of Ro60 cDNA was successful. Lane 5: 500bp DNA ladder. Lanes 3 and 8: pET8c vector. Lanes 1 and 10: NcoI/BamH1 digestion of pET8c showing Ro60 DNA.](image-url)
Figure 3.2: The pMAL vector was isolated from E.coli TB1 culture using the lithium chloride plasmid purification method so that the Ro60 cDNA could be ligated in to this vector. Electrophoresis on a 0.7% (w/v) agarose gel of the pMAL plasmid preparation confirms the plasmid isolation was successful. Lane 3: 500bp DNA ladder. Lane 7: pMal vector. Multiple bands seen for pMAL may represent different conformations, open circular, supercoiled and closed circular of the plasmid; formed during the isolation procedure that would migrate at different rates.

Figure 3.3: Prior to cloning of the Ro60 DNA insert into the pMAL vector the Ro60 cDNA was amplified by PCR (The PCR primers were synthesised with EcoRI and XbaI restriction sites on the 5' and 3' ends respectively, which when used for PCR would facilitate the cloning of the Ro60 DNA insert into the pMAL vector). Agarose gel electrophoresis confirmed the amplification of Ro60 cDNA. Lane 2: 500bp DNA ladder. Lanes 4 and 6: PCR product. Lane 7: no template control. Lane 8: no primer control.
3.4.3 Transformation with recombinant plasmid

E.coli TB1 was transformed with plasmid DNA from the ligated pMAL vector and PCR insert. Plasmid DNA mini-preparations from random transformed colonies (n=6) were digested with EcoRl/Xba1 (Figure 3.4: Lanes 2-7) to identify those containing the Ro60 insert. Three clones were shown to contain the Ro60 insert (Figure 5: Lanes 4, 6 and 7).

![Figure 3.4: EcoRl/Xba1 digests of the pMAL vector isolated from transformed E.coli TB1 colonies. These were performed to excise Ro60 cDNA from the pMAL vector to see if the ligation reaction had been successful and identify which transformed colonies may contain the Ro60 recombinant plasmid. Lanes 2-7: EcoRl/Xba1 digest of pMAL vector DNA from six random transformed colonies. Lanes 4, 6 & 7 contain DNA fragment of the same size as Ro60 (1.6kb) and were suggested as colonies containing the Ro60 recombinant plasmid. Lane 9: 500bp DNA ladder.]

3.4.4 Overexpression of MBP-Ro60 fusion protein

Glycerol stocks were made of the three positive clones and one was chosen for IPTG induced overexpression of Ro60/Mal fusion. Different IPTG concentrations (0.1, 0.5 and 1mM) used showed no difference in amount of overexpressed protein (data not shown). The cells were overexpressed for 4hr and 24hr using a 1 in 100 dilution of starter culture pre-induction (Figure 3.5: Lanes 4 and 5 respectively) and a 1 in 20 dilution of starter culture (Figure 3.5: Lanes 8 and 9 respectively). Uninduced cells (1 in 100 dilutions) are shown in Figure 3.5: Lanes 1 and 2. Overexpression with and without glucose in the medium was also investigated using a 1 in 100 dilution of starter culture for 4 and 24hr (Figure 3.5: Lanes 6 and 7) as it was suggested to include glucose in the media to repress amylase expression, as this may interfere with binding of the fusion protein to amylose resin during affinity chromatography (New England Biolabs, product information). The optimum conditions for overexpression were shown to be a 1 in 100 dilution of starter culture and 4hr incubation (Figure 3.5: Lane 4).
3.4.5 Determination of solubility of overexpressed MBP-Ro60 fusion protein

Using optimised conditions for overexpression, experiments were carried out to determine if the overexpressed protein was present in the soluble or insoluble fraction of cells following cell lysis and any Ro60/mal protein present in the soluble fraction was tested for solubility. The results were investigated by SDS-PAGE, overexpressed Ro60/mal (106kDa) was shown to be present in the induced cells before and after cell lysis (Figure 3.6: Lanes 3 and 4). The cell supernatant was separated from the insoluble fraction by centrifugation at 10,000×g for 10min (Figure 3.6: Lanes 5 and 6) and the majority of the overexpressed protein was shown to be present in the resuspended insoluble fraction (Figure 3.6: Lane 6). The cell supernatant (and insoluble fraction) was centrifuged at 100,000×g for 45min (Figure 3.6: Lanes 7 and 8). This was to test the solubility of any protein in the cell supernatant and it demonstrated that the Ro60/mal fusion was not soluble when overexpressed at 37°C.

The solubility of overexpressed protein has been shown to be increased when overexpression was performed at a lower temperature (Schein and Notebom, 1988). This was investigated by Ro60/mal overexpression at 15°C. Again the results were investigated by SDS-PAGE. Overexpressed Ro60/mal (106kDa) was shown to be present in the induced cells before and after cell lysis (Figure 3.7: Lanes 3 and 4). No Ro60/mal was present in the uninduced cells (Figure 3.7 Lane 2). The cell supernatant was separated from the insoluble fraction by centrifugation at 10,000×g for 10 minutes (Figure 3.7: Lanes 5 and 7) and this time of the overexpressed protein was shown to be present in both the cell supernatant and resuspended insoluble fraction (Figure 3.7: Lanes 5 and 7). The cell supernatant was centrifuged at 100,000×g for 45min (Figure 3.7: Lane 6) to test the solubility of the Ro60/mal in the cell supernatant and again it demonstrated that the Ro60/mal fusion was still not soluble when overexpressed at a reduced temperature (Figure 3.7: Lane 6). This indicated that the Ro60/Mal fusion could be expressed as inclusion bodies.
Figure 3.5: Trial overexpressions of *E. coli* TB1+pMAL/MBP-Ro60 under different culture conditions (dilution of starter culture, induction time and glucose supplementation) to investigate optimum conditions for overexpression. Samples of the overexpressed cells were separated using 10% (w/v) SDS-PAGE. For all overexpressions *E. coli* TB1+pMAL/MBP-Ro60 cells were induced with 0.1mM IPTG. Lane 1: SDS-PAGE precision prestained standards. Lane 2: uninduced cells, 1 in 100 dilution, 4hr. Lane 3: uninduced cells, 1 in 100 dilution, 24hr. Lane 4: induced cells, 1 in 100 dilution, 4hr. Lane 5: induced cells, 1 in 100 dilution, 24hr. Lane 6: induced cells, 1 in 100 dilution, 4hr, with 0.2% glucose. Lane 7: induced cells, 1 in 100 dilution, 24hr, with 0.2% glucose. Lane 8: induced cells, 1 in 20 dilution, 4hr. Lane 9: induced cells, 1 in 20 dilution, 24hr. Lane 4: the optimum conditions for overexpression were shown to be a 1 in 100 dilution of starter culture and 4hr incubation.

Figure 3.6: Overexpression of *E. coli* TB1+pMAL/MBP-Ro60 induced with 0.1mM IPTG for 4 hours (1 in 100 dilution of starter culture) at 37°C to determine if the overexpressed protein was present in the soluble or insoluble fraction of cells following cell lysis. Samples of the overexpressed cells and lysed cells were separated using 10% (w/v) SDS-PAGE. Lane 1: SDS-PAGE precision prestained standards. Lane 2: uninduced cells. Lane 3: induced cells. Lane 4: lysed cells. Lane 5: lysed cell supernatant. Lane 6: lysed cell insoluble fraction. Lane 7: supernatant from lysed cell supernatant centrifuged at 100,000×g, 45min to test for solubility. Lane 8: insoluble fraction from centrifuged lysed cell supernatant. The majority of the overexpressed protein appears insoluble and is present in the resuspended insoluble fraction (Lane 6).
Figure 3.7: Overexpression of *E. coli* TB1+pMAL/MBP-Ro60 induced with 0.1mM IPTG for 4 hours (1 in 100 dilution of starter culture) at 15°C to determine if the overexpressed protein was present in the soluble or insoluble fraction of cells following cell lysis. Samples of the overexpressed cells and lysed cells were separated using 10% (w/v) SDS-PAGE. Lane 1: SDS-PAGE precision prestained standards. Lane 2: uninduced cells. Lane 3: induced cells. Lane 4: lysed cells. Lane 5: lysed cell supernatant. Lane 6: supernatant from lysed cell supernatant centrifuged at 100,000×g, 45 minutes to test for solubility. Lane 7: insoluble matter resuspended from lysed cells. Overexpressed protein present in the lysed cell supernatant (Lane 5) was not retained in the supernatant when tested for solubility (Lane 6).

### 3.4.6 Isolation of inclusion bodies

A general protocol was used to isolate inclusion bodies from IPTG induced Ro60/Mal overexpression (Marston, 1987). Samples were taken during the procedure for SDS-PAGE analysis as inclusion bodies may be solubilised by detergents used during cell lysis and steps included to wash the isolated inclusion bodies. Ro60/Mal was overexpressed, the cells were lysed and Ro60/Mal was detected in the cell lysis supernatant (Figure 3.8: Lane 2). However, this protein was not retained in the soluble fraction following centrifugation at 100,000×g for 45 minutes (Figure 3.8: Lane 3) indicating that it was not soluble. Ro60/Mal was also detected in the insoluble (inclusion body) fraction (Figure 3.8: Lane 4). This fraction was washed and some Ro60/Mal was detected in the wash buffer (Figure 3.8: Lane 5), this was not tested for solubility. On completion of the inclusion body isolation it was confirmed that the Ro60/Mal protein was overexpressed as inclusion bodies (Figure 3.8: Lane 7).
Figure 3.8: An inclusion body preparation from overexpressed E. coli TB1+pMAL/MBP-Ro60 was performed to investigate if the Ro60/mal fusion was expressed as inclusion bodies. Samples were taken at different stages of the method and separated by SDS-PAGE to investigate if Ro60/Mal was solubilised during the procedure. Lane 1: SDS-PAGE precision prestained standards. Lane 2: cell lysis supernatant. Lane 3: cell lysis supernatant centrifuged at 100,000×g, 45 minutes. Lane 4: cell lysis insoluble fraction. Lane 5: wash buffer of cell lysis insoluble fraction. Lane 7: Ro60/Mal isolated inclusion bodies.

3.4.7 Binding of Ro60/mal fusion to amylose affinity resin

Affinity chromatography was to be used to purify Ro60/Mal had it been produced as a soluble protein, this was to be done using the affinity of Mal for amylose resin. The Ro60/mal fusion protein in the cell lysis supernatant of cells overexpressed at 15°C, although not soluble, was still tested for its ability to bind amylose resin. Ro60/Mal was overexpressed at 15°C and the results were investigated by SDS-PAGE, overexpressed Ro60/Mal (106kDa) was shown to be present in the induced cells before and after cell lysis (Figure 3.9: Lanes 3 and 4). Ro60/Mal was not present in the uninduced cells (Figure 3.9 Lane 2). The cell supernatant was separated from the insoluble fraction by centrifugation at 10,000×g for 10min and the overexpressed protein was shown to be present in both the cell supernatant and resuspended insoluble fraction (Figure 3.9: Lanes 5 and 7). Ro60/Mal in the cell supernatant shown to be able to bind to amylase affinity resin (Figure 3.9: Lane 7) but not all the protein was able to bind and the unbound protein was retained in the cell supernatant (Figure 3.9: Lane 8).

After purification of Ro60/Mal using the affinity of Mal for amylose resin Ro60 was to be cleaved from the Mal fusion using the enzyme Factor Xa. The ability of Factor Xa to cleave Ro60/Mal was investigated. The Ro60/Mal bound to the amylose resin was eluted with lysis buffer containing 10mM maltose and concentrated using a microcon protein concentrator (Figure 3.10: Lane 2). Factor Xa was used to cleave the Ro60/Mal fusion and a Ro60 (60kDa) and maltose binding protein...
(43kDa) were produced, not all the Ro60/Mal was cleaved and a band of 102kDa was still present (Figure 3.10: Lane 3). A band at 50 kDa was also identified this may represent a possible a site in Ro06 that could be cleaved by factor Xa.

Figure 3.9 E.coli TB1+pMAL/MBP-Ro60 was overexpressed at 15°C and the Ro/Mal fusion protein present in the cell lysis supernatant (although previously shown to be insoluble) was tested for its ability to bind amylose resin. Samples taken to confirm overexpression and the Ro60/mal fusion binding to amylose resin were separated by SDS-PAGE. Lane 1: SDS-PAGE precision prestained standards. Lane 2: uninduced cells. Lane 3: induced cells. Lane 4: lysed cells. Lane 5: cell lysis supernatant. Lane 6: cell lysis insoluble fraction. Lane 7: Ro60/mal fusion in cell lysis supernatant bound to amylose resin. Lane 8: protein from cell lysis supernatant failing to bind to resin.

Figure 3.10: To obtain the 60kDa Ro protein Factor Xa was used to cleave the Ro/Mal fusion protein (eluted from amylose resin). The products of the reaction were separated by SDS-PAGE. Lane 1: SDS-PAGE precision prestained standards. Lane 2: concentrated Ro60/mal fusion protein eluted from amylose resin. Lane 3: Ro60 and maltose binding protein cleaved by factor Xa. Ro60 was also overexpressed using the original pET8c vector as an alternative way to obtain recombinant Ro60 since previous experiments demonstrated the Ro/Mal did not to have enhanced solubility. Overexpressed Ro60 using the pET8c vector was shown to be present as inclusion bodies. Lane 4: Ro60 purified from overexpression in E.coli BL21(DE3)pLysS+pET8c/Ro60
3.4.8 Overexpression of Ro60 in E.coli BL21 (DE3) pLysS+pET8c/Ro60

It was decided that as the Ro60/Mal fusion protein was not soluble, Ro60 would be overexpressed and purified using the original vector (pET8c). The purified inclusion bodies were solubilised using 6M Guanidine-HCl and a sample visualised by SDS-PAGE (Figure 3.10: Lane 4)). The protein solution was diluted, as protein concentrations of 40-80 µg/ml have been shown to be favourable for renaturation. For renaturation to occur the Guanidine-HCl was removed using gradient dialysis against PBS (Figure 3.11: Lane 4) as well as a simpler method of diluting the solubilised inclusion bodies prior to dilution in PBS. (Figure 3.11: Lane 8). The samples were tested for solubility by centrifugation at 100,000×g for 45 minutes and a sample of the supernatant taken for SDS-PAGE (Figure 3.11: Lane 6 and 10). Ro60 was isolated to high purity and was soluble using either gradient dialysis or dilution in PBS to promote refolding (Figure 3.11). The micro protein assay using the Pierce BCA reagent was used instead of the standard Bradford assay to measure the protein concentration of the diluted protein which was expected to be ~ 50µg/ml. The actual concentration was only 8µg/ml. This was of sufficient concentration for Western blot analysis to identify the protein as Ro60 with anti-Ro antibody but protein concentration was required for further experiments.

![Figure 3.11: Ro60 purified from overexpression in E.coli BL21(DE3)pLysS+pET8c/Ro60. Isolated inclusion bodies were dissolved in 6M Guanidine-HCl the protein was then renatured by methods of dilution and dialysis with PBS and tested for solubility. Purified protein was separated by 10% (w/v) Tris-Glycine SDS-PAGE gel of purified Ro60 visualised using silver staining. Lane 2: molecular weight markers. Lane 4: Ro60 renatured by gradient dialysis. Lane 6: supernatant after centrifugation at 100,000×g for 45 minutes of Ro60 renatured by gradient dialysis. Lane 8: Ro60 renatured by dilution in PBS. Lane 10: supernatant after centrifugation at 100,000×g for 45 minutes of Ro60 renatured by dilution in PBS.](image-url)
3.4.9 Optimisation of Ro60 Western blotting

It was confirmed that the isolated protein was Ro60 by Western blotting with anti-Ro60 antibodies. Commercial anti-Ro positive sera (Serologicals) were used and these were labelled as anti-Ro1, anti-Ro2 and anti-Ro/La. These antibodies were tested using a dilution of 1:500 in 5% blocking reagent and anti-Ro/La gave positive Ro60 immunostaining using these conditions (Figure 3.12:C). For immunoblotting with anti-Ro2 a blocking concentration of 10% was also used as previous results had shown high background staining. This may have been due to non-specific binding of proteins in the anti-Ro positive sera to the membrane; a higher blocking concentration may have helped to reduce background staining. No Ro60 immunostaining was seen with this antibody under either of these conditions (Figure 3.12:A and D) and a higher concentration of Ro60 may be required for antibody detection. The non-specific staining with anti-Ro1 was very high (Figure 3.12:B) and this could be reduced by using a higher percentage of blocking reagent or perhaps using less antibody.

![Western blot diagram](image)

Figure 3.12: Purified Ro60 was detected with commercial anti-Ro positive sera by Western blotting to confirm its identity. Different anti-Ro positive sera were used to optimise the detection of Ro60. A: anti-Ro2 primary antibody (1°) blocked with TBS/T containing 5% (w/v) dried skimmed milk (5% blocking reagent). B: anti-Ro1 1° with 5% (w/v) blocking reagent. C: anti-Ro/La 1° with 5% (w/v) blocking reagent. D: anti-Ro1 1° with 10% (w/v) blocking reagent.

It was decided to use anti-Ro/La for future immunoblotting and different antibody dilutions and blocking conditions were tested to find the optimum conditions for immunoblotting with this antibody. Using less primary antibody was shown to reduce background staining (Figure 3.13: A and B) and also using less secondary antibody (Figure 3.13: A and D) but increasing the blocking concentration did not reduce the background (Figure 3.13: A and C). It was decided to keep the primary antibody concentration at 1:500 but to use less secondary 1:20000 and to use blocking reagent to dilute the antibodies. This showed sensitive detection of Ro60 with minimum background (Figure 3.13:E).
Figure 3.13: Ro60 was detected with anti-Ro positive sera labelled as anti-Ro/La antibody. The method was optimised using this antibody by using different concentrations of both the primary (1°) and secondary antibody (2°) and also different concentration of blocking reagent. A: 1° - 1 in 500 dilution (1:500); 5% (w/v) blocking reagent; secondary antibody (2°) -1:10000. B: 1° -1:1000; 5% (w/v) blocking reagent; 2° -1:10000. C: 1° -1:500; 7.5% (w/v) blocking reagent; 2° -1:10000. D: 1° -1:500; 5% (w/v) blocking reagent; 2° -1:20000. E: 1° -1:500; 5% (w/v) blocking reagent; 2° -1:10000. For the final blot antibodies were diluted with 5% (w/v) blocking reagent. Antibodies in blots A-D were diluted with TBS/T

3.4.10 Concentration of Ro60 solution in PBS

Several methods of concentrating Ro60 were tried in an attempt to concentrate the protein that is known to 'precipitate' from solution at higher concentrations. These included centrifugal concentrators, stirred cell concentrators and freeze-drying. High molecular weight Polyethyleneglycol (PEG) was used to gently draw fluid from the protein solution in dialysis tubing, enabling up to a 10-fold concentration (Figure 3.14: Lane 5). After protein concentration some of the protein was precipitated following centrifugation at 100,000xg for 45min to test solubility (Figure 3.14: Lane 6) but using this method concentrations were in the range of 50-100μg/ml. The SDS-PAGE gel was stained with Coomassie blue which has a detection limit of about 1μg and therefore the band in Lane 6 is just visible.
Figure 3.14: Purified Ro60 was concentrated up to 10 fold using high molecular weight Polyethylene glycol (PEG) and the concentrated protein was tested for solubility. The concentrated protein was separated by SDS-PAGE and visualised with Coomassie blue staining. Lane 4: molecular weight markers. Lane 5: concentrated Ro60 in PBS. Lane 6: supernatant of concentrated Ro60 in PBS centrifuged at 100,000×g, 45 minutes.
3.5 DISCUSSION

ROS modification of protein has been suggested as a possible mechanism leading to the development of autoantigenicity (Peng et al., 1997). The source of ROS capable of inducing oxidative damage in vivo may be cellular (Turrens, 1997) but could also involve those generated by UV in sunlight (Lawely et al., 2001). In SLE the presence of anti-Ro60 has been associated with the development of photosensitive skin lesions (Mond et al., 1989) and it could therefore be suggested that UV-induced ROS play a role in the development of the Ro60 autoantigen. In order to provide evidence for this possible mechanism the aim of this chapter was to purify human recombinant Ro60. This was to be used subsequently to generate oxidative damage to this protein and to look at responses to the modified protein by immune cells from anti-Ro positive SLE or SS subjects.

Production of recombinant Ro60 using the expression vector pET8c was known to generate inclusion bodies and the recombinant Ro60 produced was completely insoluble (G. Pruijn, University of Nijmegen, personal communication). Published methods for the purification of Ro60 from inclusion bodies of Ro60 were available (Peek et al., 1994) but it appeared almost impossible to develop a satisfactory renaturation procedure for this protein (G. Pruijn, University of Nijmegen, personal communication). An alternative way of improving the solubility of recombinant proteins has been shown to be the use of fusion proteins where the highly soluble fusion partner has been shown to improve solubility of several proteins (Kapust et al., 1999). It was therefore decided to subclone Ro60 into expression vector pMAL for the production of a Mal fusion protein. In addition to the enhanced solubility demonstrated by several other highly insoluble Mal fusion partners the affinity of Mal for amylose resin would enable Ro60 to be purified by affinity chromatography.

Ro60 was successfully recloned into the pMAL vector and initial overexpression of the Ro/Mal fusion proved to be insoluble. When the cells were overexpressed at reduced temperature (15°C), known to increase the protein solubility of unfused proteins (Bishai et al., 1987), solubility appeared to be increased as some of the overexpressed protein was observed in the soluble fraction. However, when tested for solubility by centrifugation at 100,000×g for 45 min this protein was not retained in solution. It was unfortunate that Ro60 solubility was not enhanced by this procedure but it is not possible to predict for insoluble proteins whether this technique would be beneficial. The literature had shown that Mal fusion was able to promote the solubility of all six insoluble proteins that were tested (Kapaust et al., 1999). However CATΔ9/Mal was mostly insoluble when overexpressed at 37°C but overexpression at 25°C increased its solubility (Schein and Noteborn, 1988). In these investigations solubility was assessed as protein retained the supernatant after the insoluble debris
was pelleted by centrifugation at 20,000×g (no time was stipulated). Protein solubility has been defined in the literature as that which is not sedimented by centrifugation for 30,000×g for 30min (Kinsella, 1984). A more strict criterion has been suggested (for membrane proteins) of 100,000×g for 1 hr (Hjelmeland and Chrambach, 1984). For Ro60/Mal solubility was assessed after the cell supernatant was centrifuged for 100,000×g for 45min and it is debatable whether this stricter criterion would influence the result, however, one can be sure that all insoluble protein would be pelleted at this speed.

An explanation for the inability to solubilise CATΔ 9/Mal was that CATΔ 9 was not a wild type protein and may not be capable of adopting a stably folded conformation (Robben et al, 1993). If the solubility does depend on correct folding conformation then Ro60, as with many other eukaryotic proteins, may not be able to fold in the environment of the E.coli cytoplasm (Tuggle and Fuchs, 1985). Moreover in eukaryotic cells Ro60 exists as a complex with small cytoplasmic RNA, Ro52 and La proteins among others (Pruijn et al, 1991) and these components may be necessary for the correct conformation of Ro60 to be achieved. Despite being mostly insoluble at 37°C, CATΔ9 did confer a modest resistance to chloramphenicol suggesting some protein in the soluble fraction had attained its biologically active conformation (Kapaust et al, 1999). Since the function of Ro60 is largely unknown such a test to determine if Ro60 could attain its biologically active function once it was rendered soluble was beyond the scope of this investigation; the aim here was to achieve soluble Ro60 of high purity at a concentration desired for subsequent investigations.

The Ro60/Mal fusion present in the cell supernatant of cell overexpressed at 15°C, although it was not retained in the supernatant after the solubility test, showed some affinity for amylose resin and this affinity of Mal to amylose depends on hydrogen bonds that in turn are positioned by the three-dimensional structure of the fusion protein (New England Biolabs, technical information). After affinity purification the Ro60/Mal fusion was shown to be cleaved by Factor Xa releasing proteins of 60kDa (Ro60), 42kDa (Mal) and also a band of 50kDa. This latter band may be due to cleavage by Factor Xa at secondary sites within Ro60. Correlations have been made between proteins that are unstable in E.coli and cleavage at secondary sites with Factor Xa, suggesting these proteins are in a partially unfolded state (New England Biolabs, unpublished observations). Also since Ro60/Mal was cleaved successfully in the first attempt with Factor Xa this may indicate the fusion protein is not in its folded state where the Factor Xa site may be inaccessible to cleavage as often denaturation of the fusion protein is required in order to aid cleavage by Factor Xa (Ellinger et al, 1991).
Since the expression of Ro60 as a Ro60/Mal fusion protein did not enhance the solubility of Ro60 the original pET expression vector for Ro60 purification was investigated. There was little literature, particularly in recent years, relating to the purification of Ro60. For work with recombinant Ro60, two basic methods of renaturation have been described. These were removal of denaturant by dilution (Peek et al, 1994) or by dialysis (St.Clair, et al, 1994). No reference to the insolubility of this protein was discussed but it is known to be extremely difficult to renature recombinant Ro60 especially for relatively high protein concentrations (Pruijn, personal communication). Ro60 is able to interact with nucleic acids and also is found to reside at the cell membrane under certain conditions, so a stringent protocol for isolating inclusion bodies was applied (Peek et al, 1994). This combined sonication, DNAse and lysozyme to ensure the protein could be purified from other cellular components. Ro60 specifically binds to small RNA molecules in eukaryotic cells and so RNAse was also included in the purification procedure (J. Hunt, University of Leicester, personal communication). When the cells were lysed the buffer volume was increased to minimise the interaction of the protein molecules and thus hopefully to reduce the chance of aggregation. After Ro60 inclusion bodies were isolated they were solublised in 6M guanidine chloride prior to the renaturation procedure.

The effect of the guanidine chloride denaturant was minimised by dilution as the dialysis method previously applied in our group had lead to problems with protein precipitation (W Lawley, personal communication). A common problem with aggregation occurring during the renaturation of protein is that the protein concentration needs to be very dilute; a typical range of protein concentration in the literature was 20-50µg/ml (Clarke et al, 1999). To ensure the concentration was not too high, solublilised inclusion bodies were diluted 4 times more than in the standard protocol. This 20 fold dilution also ensured all the denaturant was diluted out and residual guanidium hydrochloride was removed by overnight dialysis against PBS. When tested for solubility, the Ro60 appeared to be completely retained in the supernatant when visualised by silver stained SDS-PAGE but the protein concentration was only 8µg/ml. It was assumed that once in solution the concentration could easily be increased by concentrating the protein. For the purpose of subsequent treatments to the protein for ROS experiments the protein was purified using PBS as a buffer instead of Tris as this buffer is known not to be appropriate for ROS studies. This is because the OH\(^-\) rapidly attacks this buffer leading to a tris-derived radical and one end-product of OH\(^-\) attack on tris is formaldehyde (Shacker et al, 1991).
Attempts to concentrate this protein were problematic. The simplest way to achieve this would have been the use of centrifugal filter concentrators but the filters soon became impenetrable to the protein solvent and with a large volume to concentrate this was not a practical option. Freeze drying of the protein after exchange into a volatile buffer was suggested as an option (P. Glynn, MRC Toxicology Unit, personal communication) but the loss of volume of protein solution did not appear to be reflected in an increase in soluble protein concentration and the stability of the protein in this buffer was not known. A stirred cell concentrator was also used as this had the advantage of being able to concentrate large volumes of protein and the stirring of the solution may help to prevent protein from sticking to the membrane. Again, loss of volume of protein solution did not appear to be reflected in an increase in soluble protein concentration and the filtration rate was very slow. In a final attempt it was suggested to use high molecular weight PEG to gently draw fluid from the protein solution (P. Butler, De Montfort University, personal communication) in dialysis tubing and this enabled 10-fold concentration. Further attempts at concentration did not result in concentrations of soluble protein above 100μg/ml above which the protein appeared to precipitate out of solution. However this seemed by far the best method in terms of minimising protein precipitation and the most simple method to concentrate the protein. In conclusion, it appeared only by using the PEG method could R060 be retained in solution as the protein concentration was increased.

Efforts to identify optimal conditions for solubility of those eukaryotic proteins found to be insoluble when overexpressed in E. coli often relies on a time- and protein consuming trial and error approach. Optimal conditions may refer to a particular buffer or agent that may exert its effect by destabilising aggregates or enhancing native solubility. The list of additives which have been used as stabilising agents is extensive and may include kosmotropes, chaotropes, amino acids, sugar, polyhydric alcohols and detergents (Bondos and Bicknell, 2003). When using a protein in subsequent investigations, one must also consider whether certain buffer systems or additives will be appropriate to use for the proceeding experiments. Protein solubility is clearly an important issue when considering whether to work on a particular protein and a soluble alternative where possible should be considered.
CHAPTER 4:

GENERATION OF Ro60 OXIDATIVE DAMAGE AND ANTIOXIDANT PREVENTION
4.0 Generation of Ro60 oxidative damage and antioxidant prevention

4.1 Introduction

The protein autoantigen Ro60 in SLE has been suggested as a candidate target for UV-induced modification based on the evidence that autoantibodies to Ro are specifically deposited in the skin in SLE (Lee and David, 1989) and that the photosensitive skin rash in SLE patients has been found to correlate with the presence of autoantibody to the Ro60 protein (Mond, 1989). Also UVB irradiation of cultured human keratinocyte cells from photosensitive SLE patients has shown the Ro60 autoantigen to be relocalized to the surface of the cell (Casciola-Rosen et al, 1994) and this may help to explain how autoantibodies interact with this 'self' antigen which is normally present in the cytoplasm of the cell. Effects of UV-induced oxidative modification to Ro60 may also be caused by reactive oxygen species intermediates and experiments have shown ROS to be produced within human keratinocyte cell lines following UVB irradiation using an intracellular ROS probe (Lawley et al, 2000). In contrast to the proposed UV hypothesis, Ro60 may be modified by ROS of a cellular origin; the antigenicity of DNA for SLE anti-DNA antibodies has been shown to be increased following treatment of DNA with ROS but not after exposure to UV (Cooke et al, 1997).

When identifying potential in vivo targets for protein oxidation, a good starting point is the generation of oxidative protein products in vitro in a dose dependent manner, the attenuation of the oxidative response using antioxidants strengthens the case for the identification of a particular oxidative product. The current methodology in use to detect protein oxidation varies from immunodetection to specialised analytical techniques such as high performance liquid chromatography (HPLC), capillary electrophoresis and gas chromatography mass spectrometry (GCMS) (Griffiths, 2000). Protein carbonyls are a well-established way of detecting protein oxidation, as carbonyl derivatives have been identified on several amino acids and are generated by a diverse range of oxidative agents (Amici et al, 1989; Shacter et al, 1995; Silvester et al, 1998).

Although in vitro experiments using a purified protein are in many respects simplistic and cannot reflect the complexity of the cellular environment, they represent a model system to determine if a protein is a potential target for oxidative damage and allow the direct action of particular oxidising system to be assessed. For the detection of protein carbonyl groups following oxidation, using purified protein enables the measurement of protein carbonyls to be made free from interference of carbonyl groups generated on other cellular components, for example, DNA and lipids or those generated by addition to protein of other oxidised products, for example, malondialdehyde. However
studies on purified proteins are very simplistic compared to the situation in vivo. A major difference for Ro60 is that in the cell, the protein forms part a RoRNP complex in the cytoplasm of the cell with one of four small cytoplasmic Y RNA molecules (hY1 - hY5 RNA, hY2 RNA is a degradation product of hY1) (Pruijn et al, 1991) transcribed by RNA polymerase III and at least two other proteins Ro52 and La, also autoantigens in SLE (Maddison and Reichlin, 1977). The close association with these other macromolecules may influence the structural changes induced by oxidative damage to Ro60.

The cellular environment therefore represents a complex interplay of pro-oxidant and antioxidant activities. Oxidative damage to cellular biomolecules occurs when the balance of these activities is disturbed in favour of a pro-oxidant status under a condition known as oxidative stress (Stadtman and Berlett, 1998). Oxidative stress can cause damage to all cellular biomolecules for example DNA, proteins, and lipids (Breen and Murphy, 1995; Tamarit, 1998; Barber and Thomas, 1978). The primary targets of damage by oxidative stress are unclear and the results of these investigations depend on the type of reactive oxygen species involved and reliable methods to detect all forms of oxidative damage. For example, DNA is a primary target for damage when hydrogen peroxide is added to mammalian cells (Spencer, 1995) and another study has shown proteins to be a major target in cells resulting in the formation of protein hydroperoxides following treatment with peroxyl radicals (Gieseg et al, 2000). In order to reflect the more complex environment of the cell, the experimental approaches used to treat and detect reactive oxygen species damage to purified protein can be applied to the cell system by using lysates from cultured human cell as a comparative model to investigate reactive oxygen species damage to protein.
4.2 Aims

The overall aim was to induce oxidation of Ro60 in order to provide evidence for the possible mechanism of autoantigenicity involving protein modification. Firstly this was approached using UVB irradiation and MCO as model systems to attempt to generate oxidative damage to human purified recombinant Ro60 *in vitro*. The oxidative damage was assessed using blotting techniques by two methods i) anti-Ro antibody to probe for structural and/or antigenic changes to Ro60 associated with protein oxidation and ii) oxidation induced formation of protein carbonyl groups on Ro60. To investigate the mechanism of oxidative damage to Ro60, antioxidants were used to prevent any induced oxidative changes. An alternative approach was to use cell lysate from normal dermal fibroblasts to investigate MCO of Ro60. Firstly, anti-Ro antibody was used to identify Ro60 in a cell lysate sample by Western blotting. Then, as for purified Ro60, after MCO of cell lysate Western blotting with anti-Ro antibody was used to probe for structural changes to Ro60 associated with protein oxidation and to detect the formation of protein carbonyl groups using anti-DNP.
4.3 Methods

4.3.1 UVB irradiation and MCO

The generation of UV-induced oxidation of purified human recombinant Ro60 in vitro and oxidation of Ro60 by ROS as an indirect effect of UVB were performed as described in sections 2.4.1 and 2.4.2. ROS treatment was achieved using MCO known to generate hydroxyl radicals by a Fenton-type reaction. UVB doses were used in the range related to potential environmental exposure, dosage was defined as minimal erythemal dose (MED) where 1 MED for UVB equals approximately 40mJ/cm² for an average skin type and represents the dose of UV required for the generation of UV-induced erythema 24 hours after irradiation (Anonymous, 1992; Honigsmann and Thody, 1986). Under conditions of oxidative stress cells may become exposed to pathophysiological levels of reactive oxygen species (Curi et al., 1998; Szatrowski et al., 1991). To reflect this situation in vitro for the oxidation of Ro60, the hydrogen peroxide/copper ion MCO system was used. This was chosen as it has been shown in a recent study to induce oxidation of lens proteins, in preference to other MCO systems (Kato et al., 2001). A concentration range of hydrogen peroxide was employed based on those used in several previous in vitro studies (Spencer et al., 1995; Robinson et al., 1998).

4.3.2 Antioxidant prevention of MCO

Ro60 was treated with the hydroxyl radical scavengers mannitol and DMSO; and the metal ion chelators DTPA and DFO prior to MCO in an attempt to prevent oxidation (section 2.4.2).

4.3.3 Detection of Ro60 using immunoblotting techniques

Immunodetection techniques are highly sensitive and interactions between antibodies and their antigen are generally highly specific, therefore antibody based techniques were chosen to probe for oxidative changes to Ro60. Western blotting following denaturing PAGE was optimised for the detection of purified human recombinant Ro60 and was achieved using anti-Ro60 antibody from commercially available anti-Ro positive sera (section 3.4.9). Studies have shown that antigenicity of Ro60 is dependent on the tertiary structure of the molecule, such that antibody binding is largely lost with denaturation of the protein (Itoh et al., 1992). Therefore, in addition, Ro60 was also detected using the non-denaturing and non-resolving dot blot technique. Anti-Ro antibody was used to probe for structural and/or antigenic changes to Ro60 associated with protein oxidation using both techniques and these methods are described in section 2.4.3.
4.3.4 Immunological detection of Ro60 protein carbonyls using blotting techniques

The carbonyl assay was used to measure protein oxidation of Ro60 after oxidative insult; purified protein is an ideal starting material for this assay because lipid, carbohydrate and DNA oxidation products in whole cell extracts or biological fluids are known to cause interference in this assay. The immunological approach was used and initially the dot blotting technique was applied but after problems with this assay Western blotting was adopted as an alternative method (section 2.4.3). In the dot blotting method free DNPH in the derivatisation process tended to bind strongly to the nitrocellulose membrane leading to a false positive result in the sample blank. This problem was avoided with Western blotting as free DNPH was separated from protein bound DNPH during SDS-PAGE electrophoresis.

4.3.5 NDF cell lines and preparation of cell lysates

Normal dermal fibroblasts were isolated from adult human skin by Angie Gillies, Department of Pathology, University of Leicester, UK and used as a source of cell lysate for experiments; they were maintained in culture as described in section 2.5.1. Each time the cells were subcultured half of the cells were maintained in culture and the other half were used to prepare cell lysate as described in section 2.5.6.

4.3.6 Treatment of cell lysate with hydrogen peroxide and copper

Based on the total protein concentration determined in the cell lysate (section 2.3.21) treatment concentrations were calculated equivalent to those used for treating purified Ro60. The cell lysate was diluted to 1.5mg/ml and treated with doses 15 times the concentrations of H$_2$O$_2$ and Cu (section 2.5.3) that were used to treat Ro60 of protein concentration 100μg/ml.

4.3.7 Measurement of Ro60 and protein carbonyls in treated cell lysates

Western blot analysis was performed as described in section 2.4.3. For detection of Ro60 anti-Ro60 antibody was used and for carbonyl detection anti-DNP was used after samples had been derivatised with DNPH.
4.4 Results

4.4.1 The effect of UVB irradiation on structural and/or antigenic changes to Ro60

Ro60 in PBS was irradiated in triplicate with UVB doses of 0, 5 and 20 times the MED. The irradiated samples were blotted in duplicate onto a nitrocellulose membrane and immunostained with anti-Ro60 positive serum. The results of the dot blot (figure 4.1) showed no obvious change in immunostaining of Ro60 following irradiation with UVB and quantitation of the dot blot spots using densitometry confirmed there was no difference in immunostaining of Ro60 after UVB irradiation (Figure 4.2). The experiment was repeated and this time the samples were separated by SDS-PAGE and transferred onto PVDF before immunostaining with anti-Ro serum. The results of the Western blotting with anti-Ro60 positive serum detected a band at 60kDa, the size of 60kDa Ro60 (Figure 4.3). No fragments or aggregates were detected with this antibody after UVB irradiation. Again densitometry confirmed there was no difference in immunostaining of Ro60 after UVB irradiation (Figure 4.2).

Figure 4.1: The effect of UVB irradiation doses of 0, 5, and 20 MED (A-C) on the antigenicity of Ro60. UVB treated Ro60 was detected using an anti-Ro antibody by the non-denaturing and non-resolving dot blot technique. Three separate irradiations were carried out (1, 2 & 3) and each sample was loaded in duplicate. The 3 sets of irradiations were each loaded in a different order to control for non-uniform binding of the protein to the membrane. A1: 0 MED. B1: 5 MED. C1: 20 MED. A2: 20 MED. B2: 5 MED. C2: 0 MED. A3: 5 MED. B3: 20 MED. C3: 0 MED.
Figure 4.2: The effect of UVB irradiation doses of 5 and 20 MED on the antigenicity of Ro60. UVB irradiated Ro60 was detected using an anti-Ro antibody by dot blotting and Western blotting techniques. UVB irradiations were performed and analysed in triplicate. Values were derived from densitometry of Western blot and dot blot film. Data are expressed as means and standard deviations of the fold change from the 0 MED control.

Figure 4.3: The effect of UVB irradiation doses of 0, 5, 20 MED on structural and/or antigenic changes to Ro60. UVB irradiated Ro60 was detected using an anti-Ro antibody by the Western blotting technique following denaturing SDS-PAGE. UVB irradiations were carried out and analysed in triplicate. Lanes 1, 4 and 7: 0 MED. Lanes 2, 5 and 8: 5 MED. Lanes 3, 5 and 9: 20 MED.
4.4.2 The effect of MCO on structural and/or antigenic changes to Ro60

Ro60 was exposed to ROS generated by MCO using hydrogen peroxide and copper ions. A dose effect was produced by incubating with a range of hydrogen peroxide concentrations. Immunodetection of treated samples using anti-Ro positive serum by the dot blot technique showed a dose dependent decrease of immunostaining with increasing hydrogen peroxide concentration (Figure 4.4). Quantitation using densitometry showed a statistically significant (P=0.001) decrease following treatment with 400μM hydrogen peroxide and 20μM copper compared to the control (Figure 4.5).

![Figure 4.4:](image-url) The effect of metal catalyzed oxidation on the immunostaining of Ro60 as an indication of changes in structure or antigenicity of the protein. Metal catalysed oxidation of Ro60 induced by incubation with H₂O₂ (at concentrations of 25, 50, 100, 200 and 400 (μM)) and Cu (20μM) in all samples was detected using an anti-Ro antibody by the non-denaturing and non-resolving dot blot technique. Columns 1, 2 and 3 represent duplicate loading of 3 separate oxidations. A: No H₂O₂ or Cu. B: 25μM H₂O₂. C: 50μM H₂O₂. D: 100μM H₂O₂. E: 200μM H₂O₂. F: 400μM H₂O₂. G: No H₂O₂ or Cu.
Figure 4.5: The effect of metal catalyzed oxidation on the immunostaining of Ro60 as an indication of changes in structure or antigenicity of the protein. Metal catalysed oxidation of Ro60 induced by incubation with H₂O₂ (at concentrations of 25, 50, 100, 200 and 400 (μM)) and Cu (20μM) in all samples was detected using an anti-Ro antibody by the non-denaturing and non-resolving dot blot technique. Optical density values were derived from dot blot x-ray film analysed by densitometry. Data represent mean of duplicate dot-blot loadings for three separate metal catalysed oxidation treatments. Statistical analysis (n=3) was done using a Kruskal-Wallis (nonparametric ANOVA) with a Dunn’s multiple comparisons posttest. Statistically significant difference between 0μM H₂O₂ control sample and 400μM H₂O₂ treated sample highlighted as *, P value is 0.001.

A similar result was observed when the samples were analysed by Western blotting (Figure 4.6). The main Ro60 band was analysed by densitometry and the results expressed in Figure 4.7. An immunostained band >250kDa was observed with increasing concentrations of hydrogen peroxide with peak intensity at 200μM hydrogen peroxide (Figure 4.6: Lane 6). At higher concentrations of hydrogen peroxide this band was observed to decrease (Figure 4.6: Lanes 7 and 8). The bands of smaller molecular weight than Ro60 appear to represent degradation products of Ro60 and are visible because the main Ro60 band was overexposed in order to visualise the possible Ro60 aggregate of >250kDa in the treated samples. The main Ro60 band was shown to decrease with increasing concentrations of hydrogen peroxide (with 20μM copper) and this was confirmed by treating three different preparations of Ro60 with the highest concentration of H₂O₂ (400μM) and Cu (20μM). The Ro60 band was analysed by densitometry and the results (Figure 4.7) show the band intensity to be reduced by more than 25% in all samples.
Figure 4.6: The effect of metal catalyzed oxidation on the immunostaining of Ro60 as an indication of changes in structure or antigenicity of the protein. Metal catalyzed oxidation of Ro60 induced by incubation with H$_2$O$_2$ (at concentrations of 25, 50, 100, 200 and 400 (µM)) and Cu (20µM) in all samples was detected using an anti-Ro antibody by Western blotting following denaturing SDS-PAGE. In addition to the 60kDa band, the size of 60kDa Ro60 the anti-Ro antibody also detected a >250kDa band following Ro60 metal catalysed oxidation (n=2). Lane 2: No H$_2$O$_2$ or Cu, lane 3: 25µM H$_2$O$_2$, lane 4: 50µM H$_2$O$_2$, lane5: 100µM H$_2$O$_2$, lane 6: 200µM H$_2$O$_2$, lane 7: 400µM H$_2$O$_2$, lane 8: 800µM H$_2$O$_2$.

Figure 4.7: The effect of metal catalyzed oxidation on the immunostaining of Ro60 as an indication of changes in structure or antigenicity of the protein. Following metal catalysed oxidation of Ro60 induced by incubation with 400µM H$_2$O$_2$ and 20µM Cu the level of immunostaining using an anti-Ro antibody by Western blotting following denaturing SDS-PAGE was detected. The oxidation was performed on three samples of three different protein preparations of purified recombinant Ro60 (1, 2 and 3). Optical density values were derived from Western blot x-ray film analysed by densitometry. Data are expressed as fold change after treatment (400µM H$_2$O$_2$ and 20µM Cu) from the untreated (0µM H$_2$O$_2$) control.
4.4.3 Detection of protein carbonyls

Several methods have been reported to describe the immunodetection of protein carbonyls by Western blot (Levine et al, 1994), slot-blot (Robinson et al, 1999) and ELISA (Buss et al, 1996) techniques. With reference to these papers a method has been developed to measure the detection of protein carbonyls using the dot-blotting technique. The primary antibody concentration and the amount of antigen loaded onto the membrane were optimised and two methods for the derivatisation with DNPH were compared.

Optimisation of primary antibody concentration and protein loading

Carbonyl groups were detected on Ro60 using a polyclonal anti-DNP rabbit antibody after DNPH derivitisation. Optimisation of this antibody showed greatest discrimination between Ro60 and DNPH derivatised Ro60 at an antibody concentration of 1:5000 (Figure: 4.8 row B). The primary antibody detects DNP labelled molecules, in this case DNP labelled protein. Since DNPH may bind itself to the immuno-blot membrane, a control was set up containing DNPH only (Figure: 4.8 column 3) to control for binding of the primary antibody to any membrane bound DNPH. No carbonyls were detected in the absence of primary antibody, this demonstrated that there was no specific binding of the secondary antibody to protein on the membrane (Figure: 4.8 row D). Carbonyl groups could be detected using protein loadings as low as 0.3µg per dot and the staining intensity was shown to increase with protein loading up to approximately 3µg (Figure: 4.9). For further blots approximately 1µg was loaded.
Figure 4.8: Detection of carbonyl groups on purified recombinant Ro60. Ro60 was derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP by dot blotting. Different antibody concentrations were investigated (A: 1 in 50,000 dilution of primary antibody; B: 1:5000 dilution of primary antibody; C: 1:500 dilution of primary antibody) to identify the optimum concentration for immunostaining with the maximum discrimination between the derivatised Ro60 and the underivatised control (1: Ro60 alone. 2: Ro60 derivatised with DNPH). Since DNPH may bind non-specifically to the PVDF membrane a DNPH only control (3: DNPH alone) was also included. Immunostaining without the primary antibody was performed to control for non-specific binding of the secondary antibody (D: no primary antibody).

Figure 4.9: Detection of carbonyl groups on purified recombinant Ro60. Ro60 was derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP by dot blotting. Different amounts of protein were loaded onto the membrane to assess linearity and limit of detection for the assay (A1: 3.4μg Ro60. A2: 1.7μg Ro60. A3: 0.62μg Ro60. A4: 0.34μg Ro60). Ro60, which had not been derivatised, was used as a negative control (B: Ro60 alone). Since DNPH may bind non-specifically to the PVDF membrane a DNPH only control was also included (C: DNPH alone) was also included.
Comparison of sample and membrane methods of derivatisation

Methods have been described previously for the measurement of carbonyl groups in which DNPH derivatisation is performed on samples after they have been applied to the nitrocellulose membrane. This is a much simpler method if large numbers of samples are to be processed, and this approach was compared to derivatisation of the samples before they were applied to the membrane. Derivatisation of samples before blotting on to the membrane was shown to give a better discrimination between Ro60 and DNPH derivatised Ro60 (Figure 4.10) than when Ro60 was derivatised on the membrane (Figure 4.11) and therefore the process of derivatisation of the sample with DNPH before blotting on to the membrane was continued. For membrane derivatisation a separate DNPH only control was not appropriate as during the derivatisation all areas of the membrane would be in contact with DNPH and those without protein bound would act as a control for DNPH only. To discriminate any primary antibody detection of membrane bound DNPH from non-specific binding of the secondary antibody a no primary control was included in this experiment (Figure 4.11: column 2).

Figure 4.10: Detection of carbonyl groups on purified recombinant Ro60. Ro60 samples were derivatised with DNPH prior loading onto the membrane and DNP-bound protein carbonyl groups were detected using anti-DNP by dot blotting (B). Ro60, which had not been derivatised, was used as a negative control (A: Ro60 alone). Since DNPH may bind non-specifically to the PVDF membrane a DNPH only control was also included (C: DNPH alone) was also included.

Figure 4.11: Detection of carbonyl groups on purified recombinant Ro60. Ro60 was bound to the membrane and then derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP by dot blotting (3). Ro60, which had not been derivatised, was used as a negative control (1: Ro60 alone). Since DNPH may bind non-specifically to the PVDF membrane a DNPH only control was also included (C: DNPH alone). Immunostaining without the primary antibody was performed to control for non-specific binding of the secondary antibody (2: no primary antibody).
The binding of the primary antibody to the underivatised Ro60 resulting in background staining prompted trial of an alternative antibody, a polyclonal anti-DNP-KLH rabbit IgG antibody, used at the same concentration as that of the previous antibody. With this antibody there was no cross reactivity with non-derivatised Ro60 (Figure 4.12: G Ro60 control) and was used for future experiments.

4.4.4 The effect of MCO on Ro60 detected by formation of protein carbonyls

The use of anti-Ro antibody to probe for structural and/or antigenic changes to Ro60 following MCO demonstrated a significant loss of immunostaining following treatment (Figure 4.4) indicating that MCO was inducing oxidative damage to Ro60. The same method to induce oxidative damage was used in the immunodetection of protein carbonyls used to confirm oxidative changes to Ro60. Three individual treatments were carried out with 0, 25, 50, 100, 200 and 400µM hydrogen peroxide and 20µM copper and the treated samples were derivatised with DNPH and then blotted in duplicate onto nitrocellulose membranes. Carbonyl groups were detected using an anti-DNP antibody. The results of the immuno-blot (Figure 4.12) showed a dose dependent increase in protein carbonyl groups following metal catalysed oxidation and a similar effect was shown when repeating this experiment on other batches of purified Ro60 (Figure:4.14). The level of immunostaining was quantified by densitometry analysis of the exposed X-ray film (Figure 4.13) and protein carbonyl groups were show to be statistically increased following treatment with 400µM hydrogen peroxide and 20µM copper compared to the control (P=0.05).
Figure 4.12: Detection of carbonyl groups on purified recombinant Ro60 following oxidation using the H$_2$O$_2$/Cu metal catalysed oxidation system by dot-blotting. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. The oxidative dose was varied by using 25, 50 100 200 and 400μM hydrogen peroxide and 20μM copper. Oxidations were performed in triplicate and each sample was loaded in duplicate. The three treatments are denoted 1, 2 and 3 and the letters represent treatment concentrations of A: No H$_2$O$_2$ or Cu, B: 25μM, C: 50μM, D: 100μM, E: 200μM and F: 400μM of hydrogen peroxide. G: underivatised Ro60. H: DNPH alone.

Figure 4.13: Detection of carbonyl groups on purified recombinant Ro60 following oxidation using the H$_2$O$_2$/Cu metal catalysed oxidation system by dot blotting. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. The oxidative dose was varied by using 25, 50 100 200 and 400μM hydrogen peroxide and 20μM copper. Optical density values were derived from dot blot X-ray film analysed by densitometry. Statistical analysis was performed (n=3) using a Kruskal-Wallis (nonparametric ANOVA) with a Dunn’s multiple comparisons posttest. Statistically significant difference between control (0μM) and Ro60 treated with 400μM H$_2$O$_2$ and Cu highlighted as *, P value is 0.05.
Figure 4.14: Detection of carbonyl groups on 4 different preparation of purified recombinant Ro60 denoted 1 (100μg/ml), 2 (50μg/ml), 3 (30μg/ml), and 4 (90μg/ml) following oxidation using the H₂O₂/Cu metal catalysed oxidation system by dot blotting. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. The oxidative dose was varied by using 25, 50, 100, 200 and 400μM hydrogen peroxide and 20μM copper. The letters represent treatment concentrations of A: No H₂O₂ or Cu, B: 25μM, C: 50μM, D: 100μM, E: 200μM and F: 400μM of hydrogen peroxide.

For further investigations into the formation of carbonyl groups following MCO of Ro60 the dot blotting technique was replaced by the technique of Western blotting after intermittent problems with this assay where free DNPH in the derivitisation process tended to bind strongly to the nitrocellulose membrane leading to a false positive result in the sample blank. Western blotting avoided this problem as free DNPH was separated from protein bound DNPH during SDS-PAGE electrophoresis. The method adopted (Schacter, 2000) deviated from the regular SDS-PAGE protocol in that samples were derivatised with DNPH and were not heated with DTT prior to gel loading. Heating was not applied as the stability of protein-bound hydrazones to heating was not known and it was suggested that DTT alone is sufficient for the reduction of protein without the heating step enabling separation of proteins by SDS-PAGE. This approach was investigated for the separation of Ro60 where Figure 4.15 shows triplicate samples of Ro60 treated without DTT or heating, with DTT treatment but without heating and with heating and DTT treatment. The results demonstrated that the heating step in addition to DTT was not necessary for the separation of Ro60 by SDS-PAGE electrophoresis. Samples treated with DTT without heating showed separation of the 60kDa Ro60 band (Figure 4.15: 4-6) as would normally be observed with DTT treatment and heat (Figure 4.15: 7-9), however, those samples not treated with DTT or heating failed to separate Ro60 as a 60kDa band (Figure 4.15: 1-3).
Figure 4.15: The effect of heating with DTT on the separation on DNPH derivatised Ro60 by SDS-PAGE. In the carbonyl Western blot procedure the heating the step prior to SDS-PAGE was omitted. 10% (w/v) Tris-Glycine SDS-PAGE gel of triplicate samples of purified Ro60 visualised using silver staining showed that as long as DTT was included in the samples the heating step was not essential for protein separation. Lanes 1-3: no DTT or heat treatment. Lanes: 4-6: DTT but no heat treatment. Lanes 7-9: DTT and heat treatment.

Western blotting was used to detect protein carbonyls after treatment of Ro60 with 0, 25, 50, 100, 200, 400 and 800μM hydrogen peroxide and 20μM, the results of a representative blot are shown in Figure 4.16. No carbonyls were present on Ro60 without treatment (Lane: 2). After treatment of Ro60 with 25, 50 and 100μM hydrogen peroxide with 20μM copper, protein carbonyls were detected on the Ro60kDa band (Lanes: 3, 4 and 5). At treatment concentrations of 200, 400 and 800μM hydrogen peroxide and 20μM copper the level of protein carbonyls detected on the 60kDa Ro60 band were decreased with increasing hydrogen peroxide concentration or due to decreases in Ro60 “monomer”. At these concentrations of hydrogen peroxide, protein carbonyls were also detected on higher molecular weight bands, assumed to be aggregated Ro60 since the preparation was purified Ro60 and the presence of carbonyl groups on these bands increased with increasing concentrations of hydrogen peroxide (Lanes: 6, 7and 8).
Figure 4.16: Detection of carbonyl groups on purified recombinant Ro60 following oxidation using the H$_2$O$_2$/Cu metal catalysed oxidation system by Western blotting. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. The oxidative dose was varied by using 25, 50 100 200 and 400µM hydrogen peroxide and 20µM copper. Lane 1: SDS-PAGE precision prestained markers. Lane 2: 0µM. Lane 3: 25µM. Lane 4: 50µM. Lane 5: 100µM. Lane 6: 200µM. Lane 7: 400µM. Lane 8: 800µM. In addition to the 60kDa Ro60 carbonyl groups were also detected bands >250kDa following metal catalysed oxidation suggesting the treatment caused to protein to aggregate. The blot represents one of two experiments.

4.4.5 The effect of MCO on the reduction of Ro60 by dithiothreitol

The results of protein carbonyl detection by Western blotting following MCO of Ro60 indicated that at doses of hydrogen peroxide greater than 200µM, Ro60 aggregation was occurring. It was shown previously that Ro60 was aggregated in the absence DTT and this lead to the question as to whether the Ro60 aggregate was present because the oxidative conditions present during the treatment were inhibiting the reducing capacity of the DTT. The MCO process involved the incubation of Ro60 with hydrogen peroxide and copper at 37°C for 1hr. DTT was added after this incubation and thus any effects of MCO treatment of DTT would take effect from this point. Therefore treatment of Ro60 with hydrogen peroxide and copper without incubation would enable this question to be answered. A control was included with incubation of Ro60 in the absence of hydrogen peroxide and copper to exclude the possibility that incubation of Ro60 was inducing its aggregation. The results of this experiment shown in figure 4.17 demonstrated that the aggregate was present after MCO of Ro60 for 1hr at 37°C (Figure 4.17 lanes 4-6). The incubation period alone did not cause aggregation (Figure 4.17 lanes 1-3) and the reducing capacity of DTT was not affected by oxidising properties of the
MCO system (Figure 4.17 lanes 7-9). Ro60 carbonyls were detected in each experimental group, quantified by densitometry analysis of the exposed X-ray film (Figure 4.18) Statistical analysis performed using a Kruskal-Wallis (nonparametric ANOVA) test showed no difference in the levels of carbonyls between the three groups.

Figure 4.17: The effect of metal catalyzed oxidation on the reducing capacity of DTT. Ro60 aggregation was induced by oxidation with 800µM hydrogen peroxide and 20µM copper for 1hr at 37°C on triplicate samples (Lanes 4, 5 and 6) and detected by carbonyl formation by Western blotting. Incubation for 1hr at 37°C only on triplicate samples was shown not to induce aggregation (Lanes 1, 2 and 3). By omitting the 1hr incubation at 37°C essential for metal catalysed oxidation induced aggregation, the effect of 800µM hydrogen peroxide and 20µM copper on the reducing capacity of DTT could be investigated. Triplicate samples showed DTT in the presence of 800µM hydrogen peroxide and 20µM copper was still able to reduce the Ro60 protein as it was separated as a 60kDa band (Lanes 7, 8 and 9 Ro60 treated with 800µM H₂O₂ and 20µM Cu without incubation).

Figure 4.18: The effect of treatment of Ro60 with 800µM hydrogen peroxide and 20µM copper with and without incubation at 1hr for 37°C on the level of oxidation induced carbonyl groups detected by Western blotting. Values are means and standard deviations calculated from optical density values derived from densitometry analysis of exposed X-ray film. Statistical analysis of triplicate samples showed no difference in the level of carbonyl groups following of treatment of Ro60 with 800µM hydrogen peroxide and 20µM copper with or without incubation at 1hr for 37°C.
4.4.6 Prevention of MCO of Ro60 with the use of antioxidants

The treatment of Ro60 with at least 200μM hydrogen peroxide and 20μM copper showed an increase in Ro60 carbonyl groups present on an aggregate of Ro60. To further demonstrate that these effects were the result of oxidative damage an attempt to prevent these effects by the pre-treatment of Ro60 with antioxidants was investigated. Ro60 was pre-treated with the metal ion chelators, DTPA and DFO and the hydroxyl radical scavengers mannitol, DMSO and ascorbic acid. Pre-treatment with PBS was used as a control and the samples were subject to MCO using the highest concentration of hydrogen peroxide (800μM+20μMCu²⁺) previously tested, to maximise the oxidative response and samples without treatment were used as controls. All samples were analysed by Western blotting for the detection of carbonyl groups and representative blots are shown in Figures 4.19 and 4.20. The native Ro60 is difficult to visualise due to the lower level of oxidation. The blots show that carbonyl formation and aggregation are completely prevented when Ro60 is treated with the metal ion chelator DTPA (Figure 4.19: Lane 5) and partially prevented by pre-treatment with DFO (Figure 4.19: Lane 7) which is more effective as an iron chelator but can also chelate copper ions. Pre-treatment of Ro60 with hydroxyl radical scavengers, mannitol, DMSO and ascorbic acid were not able to prevent carbonyl formation or aggregation following MCO (Figure 4.20). Again the native Ro60 is difficult to visualise due to the lower level of oxidation.

![Western blot showing the detection of Ro60 carbonyl groups following pre-treatment of Ro60 with metal ion chelators and then oxidation with 800μM hydrogen peroxide and 20μM copper. Lane 1: SDS-PAGE precision prestained markers. Lanes 2 and 3: no pre-treatment of Ro60. Lanes 4 and 5: pre-treatment with DTPA. Lanes 6 and 7: pre-treatment with DFO. Lanes 2, 4 and 6: no treatment with hydrogen peroxide and copper. Lanes 3, 5 and 7: treatment with hydrogen peroxide and copper. The blot represents one of two experiments.](image-url)
**Figure 4.20:** Effect of free radical scavengers on Ro60 oxidation and aggregation. Western blot showing the detection of Ro60 carbonyl groups following pre-treatment of Ro60 with free radical scavengers and then oxidation with 800μM hydrogen peroxide and 20μM copper. Lane 1: SDS-PAGE precision prestained markers. Lanes 2 and 3: no pre-treatment of Ro60. Lanes 4 and 5: pre-treatment with mannitol. Lanes 6 and 7: pre-treatment with DMSO. Lanes 8 and 9: pre-treatment with ascorbic acid. Lanes 2, 4, 6 and 8: no treatment with hydrogen peroxide and copper. Lanes 3, 5, 7 and 9: treatment with hydrogen peroxide and copper. The blott represents one of two experiments.

### 4.4.7 Measurement of protein carbonyl groups on UVB treated Ro60 by Western blotting

The carbonyl Western blot assay was also applied to detect carbonyl formation following UVB irradiation. Ro60 in PBS was irradiated in triplicate with UVB doses of 0, 5, and 20 MED and carbonyl groups were detected using the Western blot carbonyl assay. The results of the Western blot detected the presence of carbonyls only on the main Ro60 band (Figure: 4.21) and no carbonyl groups were induced on Ro60 fragments or aggregates following UVB irradiation. The optical density of the main 60kDa band was measured by densitometry analysis of the exposed film to assess the levels of carbonyl groups after UVB irradiation compared to the control. The results show that there was no change in the levels of carbonyl groups on Ro60 following UVB irradiation (Figure: 4.22). In the previous experiment the native Ro60 was difficult to visualise due to the lower level of oxidation compared Ro60 that had been exposed to MCO. In this experiment the native Ro60 was visualised because the film was exposed for a longer time.
Figure 4.21: Detection of carbonyl groups on purified recombinant Ro60 following irradiation with UVB by Western blotting. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. Triplicate samples were irradiated with dose of 0, 5 and 20 times MED. Lanes 1, 4 and 7: 0 MED. Lanes 2, 5 and 8: 5 MED. Lanes 3, 5 and 9: 20 MED. X-ray films were exposed for longer time than in previous experiments where native Ro60 was difficult to visualise due to the lower level of oxidation compared Ro60 that had been exposed to MCO.

Figure 4.22: Detection of carbonyl groups on purified recombinant Ro60 following irradiation with UVB by Western blotting. Triplicate samples were irradiated with dose of 0, 5 and 20 times MED. Lanes 1, 4 and 7: 0 MED. Lanes 2, 5 and 8: 5 MED. Lanes 3, 5 and 9: 20 MED. Values are means and standard deviations of fold change from the non irradiated control (n=3) calculated from optical density values derived from Western blot film analysed by densitometry.
4.5 MCO of Ro60 in cell lysates

4.5.1 Detection of Ro60 in cell lysate using anti-Ro sera

Anti-Ro positive sera were used to identify Ro60 in a lysate of NDF by Western blotting. Figure 4.23 shows the use of anti-Ro positive sera to identify Ro60 in a cell lysate sample. SDS-PAGE precision prestained standards (lane 10) and purified Ro60 were electrophoresed on the gel (lanes 1 and 6) to standardise the gel for the identification of an autoantigenic protein band within the cell lysate at 60kDa. After the proteins were transferred onto PVDF membrane the membrane was cut vertically down lane 5 and half the membrane was immunostained using a primary antibody known to recognise Ro and La autoantigens (lanes 1-4) and the other half an antibody known to recognise Ro autoantigen (lanes 6-10). The amount of protein loaded into lanes 6-10 was ten times more than that in lanes 1-4 due to difference in sensitivity of the two antibodies and both immunoblots were exposed for less than five minutes during ECL detection. A duplicate experiment was performed except no primary antibody was used and this blot did not show any immunostaining and demonstrated that the secondary antibody was not binding non-specifically to the lysate proteins. The blot stained with serum positive for antibodies to both Ro and La gave three immunostained bands with one being the most prominent (for a more exposed film see figure 4.24). Since the size of the autoantigens Ro60, Ro52 and La are 60kDa, 52kDa and 48kDa conclusions on which band represented Ro60 could not be made. Identification based on the molecular weight of purified Ro60 overexpressed in E.coli may be different for Ro60 expressed in human cells as posttranslational modifications may be present that may alter its apparent molecular weight when visualised by SDS-PAGE. The blot stained with serum positive for antibodies to Ro appeared to lack specificity as it stained several bands in the cell lysate and also the SDS-PAGE precision prestained standards. For this reason the serum positive for antibodies to both Ro and La was used for further experiments and to investigate oxidative damage to protein autoantigens in SLE and (not exclusively to Ro60) by exposure of cell lysate from normal dermal fibroblast to MCO.
Figure 4.23: Detection of Ro60 in normal dermal fibroblast cell lysate using commercial anti-Ro positive sera by Western blotting. Purified Ro60 was also detected to aid identification of Ro60 in cell lysate. Two different anti-Ro positive sera were used, anti Ro/La (lanes 1-4), previously shown to be a good antibody for the detection of purified Ro60 and anti-Ro (lanes 6-10) antibody not previously optimised for the detection of purified Ro60. Lysate samples were separated in triplicate (lanes 2-4 and 7-9) and one sample of purified Ro60 (lane 1 and 6) was used for each primary antibody. Precision prestained protein markers are shown in lane 10 (non-specific binding of the anti-Ro primary antibody).

Figure 4.24: Detection of Ro60 in normal dermal fibroblast cell lysate using anti-Ro/La antibody (lanes 2-4) by Western blotting. A sample of purified Ro60 was also used to aid identification of Ro60 in cell lysate (lane 1). When the prominent band was overexposed minor bands could also be visualised. As well as 60kDa Ro60, autoantigens of similar molecular weight 52kDa Ro52 and 42kDa La may be detected with anti-Ro/La antibody anti-sera and may explain the appearance of multiple bands.
4.5.2 MCO of NDF cell lysate and the effect on SLE autoantigens

The MCO system consisting of hydrogen peroxide and copper was used to treat normal dermal fibroblast cell lysate in triplicate. The treatment concentrations were equivalent to those used to treat purified Ro60 based on the total protein concentration. The treated samples were analysed by Western blotting with anti-Ro/La positive sera and the results of the immunoblot are shown in Figure 4.25. The results show no evidence of immunoreactive aggregates of SLE autoantigens but at the highest treatment concentration a 25kDa band can be seen, this represents an immunoreactive fragment generated by MCO.

![Image of Western blot](image_url)

Figure 4.25: The effect of metal catalyzed oxidation on the immunostaining with anti Ro/La antibody as indication changes in structure or antigenicity of SLE protein autoantigens present within cell lysates. Metal catalysed oxidation of cell lysate samples in triplicate was induced by incubation for 1 hr at 37°C with H₂O₂ at concentrations of 600μM (lanes 3, 6 and 9) and 1200μM (lanes 4, 7 and 10) with Cu of 300μM. These concentrations were equivalent to those used to treat purified Ro60 based on total amount of protein. Samples without treatment (lanes 2, 5 and 8) were used as a control.

5.2.3 MCO of NDF cell lysate and the detection of protein carbonyls

Normal dermal fibroblasts cell lysates in triplicate were also treated by MCO and analysed for the formation of protein carbonyls by Western blotting. The results in Figure 4.26 show at the presented exposure time no protein carbonyl were observed without treatment compared to the treated samples and the number of carbonyls increased at the highest treatment concentration. There also appeared to be the formation of high molecular weight aggregates of proteins identified by the detection of carbonyl groups in the treated samples.
Figure 4.26: The effect of metal catalyzed oxidation on carbonyl formation detected by Western blotting on proteins present within NDF cell lysates. The samples were derivatised with DNPH and DNP-bound protein carbonyl groups were detected using anti-DNP. Metal catalysed oxidation of cell lysate samples in triplicate was induced by incubation for 1hr at 37°C with H$_2$O$_2$ at concentrations of 600µM (lanes 3, 6 and 9) and 1200µM (lanes 4, 7 and 10) with Cu of 300µM. These concentrations were equivalent to those used to treat purified Ro60 based on total amount of protein. Samples without treatment (lanes 2, 5 and 8) were used as a control.
4.6 DISCUSSION

4.6.1 Introduction

ROS have been implicated in the induction of autoimmunity via the initiation of protein oxidation reactions leading to the recognition of antigen as non-"self". In SLE, the source of ROS capable of inducing damage to antigens may be cellular (Babior et al., 1997) but could also involve those generated by UV in sunlight (Lawley et al., 2001). The aim of the work in this chapter was to investigate the effect of UVB irradiation and ROS exposure on the integrity of the Ro60 autoantigen. This was done using two methods to measure protein oxidation and the type and extent of protein damage by these systems was assessed. The evidence for modification to Ro60 at pathological levels of ROS could then be used to determine a method to induce damage to Ro60 for experiments in the final part of this project, investigating if the response of PBMC cells isolated from Ro positive SLE or SS subjects to purified Ro60 is altered after it has been damaged by ROS.

The types of oxidative modifications that can occur to proteins are numerous, due to the number of different oxidising species that exist in vivo and the susceptibility of all amino acids residues to oxidative attack (Shacter, 2000). This work has focused on the oxidising agents UVB and MCO. UVB was chosen because of the association between the presence of Ro60 autoantibodies and development of photosensitive skin lesions in SLE (Mond et al., 1989); and MCO because oxidative damage may be caused by MCO when pathological levels H$_2$O$_2$ are present. This may occur following persistent activation of inflammatory cells in autoimmune diseases associated with an inflammatory state, for example, in SLE. Elevated levels of H$_2$O$_2$ and the presence of reduced transition metal ions in cells may cause excess generation of OH$^-$ radicals and this is known to be a major cause of oxidative damage to proteins (Swallow et al., 1960). There is evidence of ROS-damaged proteins in several pathologies and some examples include Alzheimer's disease (Harris et al., 1995), rheumatoid arthritis (Chapman et al., 1989), arthrosclerosis (Leeuwenburgh et al., 1997) and Parkinson's disease (Yoritaka et al, 1996).

The oxidising source, type of modification and amino acids involved are all important factors to consider when choosing which method to use to detect protein oxidation, especially as some methods require the use of specialist techniques or expensive equipment. All oxidising sources can result in the formation of protein carbonyls as well as and cross-links, aggregates and fragments (Shacter, 2000), these can be associated with several amino acids and they are the most commonly investigated markers of oxidative damage. Carbonyl groups are chemically stable and can be
detected by immunoblotting techniques following the derivatisation of the carbonyl groups with DNPH (Robinson et al., 1999). Cross-links, aggregates and fragments can be detected by SDS gel electrophoresis and again immunoblotting procedures can be used for detection but this time with antibodies reactive with native protein (Davies and Delsignore, 1987). The advantage of being able to use antibodies for detection is that they are highly specific for antigen and are far more sensitive than alternative spectrophotometric or protein staining techniques. A high sensitivity was required to detect damage to Ro60 as the protein was only retained in solution at low protein concentrations and so immunoblottting procedures were appropriate. One problem with using antibodies to detect cross-links, aggregates and fragments is that they must remain immunoreactive; the loss of the main protein band after oxidative treatment may also be used as in indicator of the formation of other oxidatively modified forms of protein which are not reactive with the antibody.

4.6.2 **The effect of UVB irradiation on Ro60**

Western blotting with anti-Ro antibodies was used to investigate if UVB irradiation to Ro60 could cause cross-links, fragments or aggregates either by generation of immunoreactive bands in addition to the main Ro60 band or by loss of the main Ro60 band to fragments or aggregates that were not immunoreactive. ROS damage may lead to changes in protein conformation or structure and this in turn may affect protein immunoreactivity (Itoh et al., 1992), therefore additional studies would be needed to investigate if changes were as a result of loss of protein or loss of immunoreactivity. The results showed no changes in antibody staining following treatment of Ro60 with 0, 5 and 20 times the MED, despite the highest dose being considerably higher than environmental exposure levels.

The above experiment was repeated using the non-denaturing and non-resolving dot blot technique as studies have shown that antigenicity of Ro60 is dependent on the tertiary structure of the molecule, such that antibody binding is largely lost with denaturation of the protein, as would be the case in denaturing SDS-PAGE (Itoh et al., 1992). However, the above experiment demonstrated that the Ro60 antibody was able to detect Ro60 with high sensitivity after SDS-PAGE. The antibody did not appear to be more sensitive to detection when using the non-denaturing dot blot technique. This may have been because the purified recombinant protein did not achieve its native state after the renaturation procedure was performed during the purification procedure. Again no changes in immunostaining were detected following irradiation with 0, 5 and 20 MED. Using this technique a decrease in immunostaining could have indicated the formation of non-immunoreactive fragments or altered of immunoreactivity following irradiation.
Protein carbonyl formation following UVB irradiation with 0, 5 and 20 MED was also performed using Western blotting. The samples were derivatised using DNPH and the DNPH bound carbonyl groups were detected using anti-DNP antibody. There was no change in the level of protein carbonyl groups following UVB irradiation any of the doses examined.

The literature for UV induced protein oxidation is not as extensive as for ROS. Singlet oxygen has often been proposed as the oxidising species in light dependent reactions. This can be generated when light absorbed by photosensitive molecules in vivo is transferred onto adjacent O$_2$ molecules (described as a type II mechanism) (Bosca et al 1997). After light absorption a photosensitiser may also directly initiate protein oxidation (type I mechanism) (Gerhardt et al, 1999). In addition illumination of several photosenitisers in solution has been reported to produce OH$^\cdot$ and O$_2^-$ suggesting that singlet oxygen is not the only ROS involved in photosensitivity reactions (Martin and Logsdon, 1987). Utilising recombinant purified proteins means that cellular photosensitisers are essentially absent from the model. Here no exogenous photosensitisers were added. Several amino acid molecules in proteins are able to absorb energy from light illumination directly or by transfer from singlet oxygen and this can result in modification of amino acids. The amino acids histidine, tryptophan and tyrosine are most susceptible as the aromatic ring is able to absorb energy from light illumination resulting in oxidative damage where the mechanism may involve type I or type II photosensitivity reactions (Matheson and Lee, 1979).

Other commonly investigated oxidation products which result are N-formylkynurenine from tryptophan and di-tyrosine from tyrosine (Finley et al, 1988; Heinecke et al, 1993). Cysteine, methionine and histidine are able to react with singlet oxygen forming amongst other products, disulphides, methionine sulfoxide and 2-oxohistidine respectively (Purdie, 1967; Amus 1979; Lewisch and Levine, 1995). SDS-PAGE would therefore be appropriate to detect formation of cross-links involving oxidation of cysteine, tyrosine and histidine (Davies and Delsignore, 1987). For histidine the mechanism of aggregation is not known but studies have shown this residue to be necessary for high molecular weight aggregates to form following singlet oxygen induced oxidation (Balasubramanian et al, 1990). None of these products were encountered following UVB irradiation of Ro60 at least not in amounts sufficient to detect by immunoblotting. The loss of tryptophan and formation of di-tyrosine could have been detected by fluorescence measurements as this technique is also very sensitive (Finley et al, 1998; Leewnburch et al, 1998) however the detection of modification on one type of amino acid is more difficult to detect and will depend on the number of these residues in the protein. For tyrosine it has been reported, that its oxidation products do not
appear to be as abundant in biological samples as protein carbonyls and the levels of di-tyrosine are particularly low (Dean et al, 1997).

The literature specifically related to UVB has shown photodamage to proteins to include cross-linking, aggregation, denaturation and degradation and a recent study has shown tryptophan photolysis leads to a UVB-induced 66kDa photoprodut of rubisco in vitro and in vivo (Gerhardt et al, 1999). Rubisco is a particularly good protein model for UV studies because it is the most abundant leaf protein and proteins in plant leaves are particularly vulnerable to UVB exposure. Other studies relating to singlet oxygen exposure of protein have been performed. Singlet oxygen was shown to oxidise tryptophan residues to N-formylkynurenine and tyrosine residues were shown to react with singlet oxygen but no modified products of the latter were detected. In the same study oxidation of histidine was shown to be necessary for high molecular weight aggregates to be formed (Balasubramanian et al, 1990). This was supported by a similar study showing singlet oxygen-induced histidine oxidation to be involved in the formation of collagen cross-links (Au and Maddison, 2000). No carbonyl groups were detected on Ro60 following UVB irradiation. If singlet oxygen is the principle mechanism by which UVB damage is induced then this may explain why carbonyl groups were not detected, as singlet oxygen has been to preferentially attack tryptophan, histidine, methionine and cysteine and has a lesser capacity to induce carbonyls (Balasubramanian et al, 1990).

4.6.3 The effect of MCO on Ro60

Metal catalysed oxidation of Ro60 was also investigated using the \( \text{H}_2\text{O}_2/\text{Cu}^{2+} \) system. The literature suggested that the mechanism involves \( \text{OH}^- \) generation at protein metal-binding sites which then oxidises amino acid side chains in the local environment of the bound metal. In contrast \( \text{OH}^- \) generation by radiolysis results in more global modification of many different amino acids and extensive fragmentation. Firstly, the immunostaining of Ro60 with anti-Ro60 using the dot blot technique following treatment with increasing doses of \( \text{H}_2\text{O}_2 \) (constant \( \text{Cu}^{2+} \)) was investigated. Immunostaining was shown to decrease with increasing concentrations of \( \text{H}_2\text{O}_2 \) and at 400 \( \mu\text{M} \) the decrease was significantly different from the control, suggesting the formation of non-immunoreactive aggregates or fragments or loss of immunoreactivity.

This treatment was repeated and Ro60 was resolved by SDS-PAGE and detected using anti-Ro antibody. A similar trend was observed i.e. loss of the 60kDa band, and a high molecular weight band was formed at the interface between the resolving and stacking gel that increased with
concentration of H$_2$O$_2$, with peak intensity at 200µM. The band could not account for the loss of Ro60 at higher concentrations of H$_2$O$_2$ as both the main band and the aggregate band decreased at concentrations higher than 200µM H$_2$O$_2$. This suggested the formation of other non-immunoreactive products or loss of immunoreactivity of the main Ro60 band or the high molecular weight aggregate due to further oxidation. The reproducibility of this effect of decreased immunostaining was confirmed across for three independent batches of purified Ro60.

Using the same treatment concentrations the formation of protein carbonyls was investigated using both dot blotting and Western blotting techniques. Ro60 carbonyl groups were shown to be increased in a dose dependent manner following treatment with H$_2$O$_2$ with Cu$^{2+}$; this effect too could be reproduced on different batches of Ro60. The effect was shown to be significantly different compared to the non-treated control only for the 400µM H$_2$O$_2$ treatment despite the high level of carbonyl formation; however the variation in the individual values at each dose resulted in high standard deviations for this assay. Variables in the assay included the rate of OH' generation in individual reactions and the high reactivity of OH' with amino acid side chains.

Interestingly, the Western blot assay showed the increased levels of carbonyls to be generated on a high molecular weight aggregate of Ro60, at the top of the stacking gel. This aggregate was not detected in previous experiments with anti-Ro (presumably non-immunoreactive) and appeared to possibly account for the loss of anti-Ro immunostaining observed in previous experiments. Carbonyls were also detected on the aggregate detected previously with anti-Ro at the interface between the stacking and resolving gel but levels were similar to those detected on the main Ro60 band at the lowest treatment of H$_2$O$_2$. Under the assay conditions used, levels of carbonyls on the untreated protein were undetectable compared to the treated samples.

The results for the carbonyl Western blot were very similar to those of Kato et al, 2001, who investigated the effects of four MCO systems on the oxidation of lens proteins. Using H$_2$O$_2$/Cu$^{2+}$ and ascorbic acid/Cu$^{2+}$ these workers observed carbonyl groups on aggregated bands at the top of the stacking gel and also at the interface between the stacking and resolving gel. With H$_2$O$_2$/Fe-EDTA and ascorbic acid/Fe-EDTA they observed carbonyls generated on aggregate bands at the interface between the stacking and resolving gel. Since the aggregated bands are present after reducing SDS-PAGE they do not represent the formation of disulphide bonds as commonly formed by the oxidation of highly oxidation-sensitive cysteine residues. Kato et al, 2001 found using an anti-
dityrosine antibody, a band at the top of the stacking gel only on the sample treated with H$_2$O$_2$/Cu$^{2+}$ and suggest that this MCO system and not the others produced the formation of dityrosine following the oxidation of lens proteins. Based on this evidence di-tyrosine may be a possible explanation for the formation of the aggregated Ro60 product. The lack of a commercial anti-dityrosine antibody meant we were unable to investigate this further at this stage.

All oxidising species are able to oxidise susceptible sulphur atoms in cysteine and methionine, however MCO is a more potent form of oxidative attack and may induce formation of carbonyls on many amino acid side chains. Histidine and tyrosine are both susceptible to MCO, leading to the formation of cross-link products (Heinecke et al., 1993; Shen et al., 1996). Carbonyls can be induced by MCO (Amici et al., 1989) and other oxidising sources but are more difficult to induce compared to modification to cysteine and methionine residues and thus reflect more severe oxidative stress (Dalle-Done et al., 2001). Elevated levels are often associated with disease-associated dysfunction, for example neurons in the brain regions most severely affected Alzheimer's disease pathology contain a significant elevation of actin carbonyl content (Aksenov et al., 2001).

4.6.4 Mechanism for MCO effects on Ro60

Original studies on MCO oxidation of protein investigated the oxidative inactivation of the enzyme glutamine synthetase (Levine et al., 1981). Given that this process was insensitive to inhibition by various free radical scavengers and that only one or at most a few amino acid residues of a given protein are modified (Samuni et al., 1983) a mechanism for the site-specific modification by metal-ion catalyzed oxidation was proposed and is illustrated in Figure 4.27. The metal ion binds to the protein and reaction of H$_2$O$_2$ with the complex leads to the formation of OH$^-$ within the local environment which rapidly then attacks the protein via abstraction of a hydrogen atom forming a carbon-centred radical. The process is described as caged due to its insensitivity to inhibition by OH$^-$ scavengers. In this diagram spontaneous hydrolysis of the amino derivative yields an aldehyde derivative and NH$_3$. 

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Figure 4.27: Possible mechanism for the metal ion-catalyzed oxidation of protein adapted from Stadtman, 1990. A complex is formed between the protein and a bound transition metal ion. This reacts with H$_2$O$_2$ to form of OH in the the local environment of the bound metal ion. OH' then rapidly attacks the protein forming a carbon-centred radical via hydrogen atom abstraction. The process is insensitive to inhibition by OH' scavengers and is described as a caged reaction. Here the spontaneous hydrolysis leads to formation of a carbonyl group on the protein and release of NH$_3$.

Whether the mechanism of damage to Ro60 does involve site specific modification has not been determined and will depend whether Ro60 is able to bind Cu$^{2+}$. In contrast, gamma-irradiation, which causes direct formation of OH' is not restricted to the metal-binding site in a protein and causes a higher degree of protein aggregation and chemical degradation than MCO. Ro60 appears to be mostly in aggregated form at doses of 400$\mu$M H$_2$O$_2$ and levels of carbonyls appear to be high (approximately 1000 fold). It may be tempting to speculate that the damage is not site specific, indeed carbonyl formation increases of only twofold to eightfold have been detected under conditions of oxidative stress in vivo (Stadtman and Berlett, 1998). However, comparison of the extent of damage cannot be made as the levels of oxidative stress will vary for each individual case. In order to help elucidate the mechanism involved for the oxidative changes to Ro60 that were observed, different types of antioxidants were used in an attempt to prevent the oxidative damage.

To investigate possible mechanisms of carbonyl formation and aggregation of Ro60, two types of antioxidants were used; OH' scavengers, mannitol and DMSO; and metal ion chelators, DTPA (chelates many metal ions) and DFO (chelates Fe$^{3+}$ ions but is also effective against Cu$^{2+}$). Ro60 was pre-treated with these antioxidants prior to treatment with 800$\mu$M H$_2$O$_2$ and 20$\mu$M Cu$^{2+}$; the dose of
was increased to ensure all Ro60 was in aggregate form. The results showed that both aggregation and carbonyl formation were not prevented using either OH' scavenger at the concentrations suggested in the literature (Kang et al, 2001; Akagawa et al, 2001; Hermes-Lima et al, 2001) However, the chelation of Cu²⁺ by DTPA did prevent both aggregation and carbonyl formation and DFO, a slightly less effective chelator of Cu²⁺, partially prevented oxidation. The inability of the free radical scavengers to inhibit the oxidative damage suggests that the H₂O₂/Cu²⁺ system did not generate a ‘free’ hydroxyl radical and this suggests a ‘caged’ reaction involving the formation of a copper-amino acid complex and the reaction of H₂O₂ with this complex causing site-specific damage to the amino acids at the binding site of the Cu²⁺ (Stadtman, 1990). An alternative explanation could be that the concentration of OH' scavengers was not high enough to scavenge all the OH' and thus prevent oxidative damage. The inhibition of damage after Cu²⁺ chelation suggests copper is required for the damage to occur and that H₂O₂ alone is not the oxidising species. The increasing damage with increasing H₂O₂ concentration suggests that H₂O₂ is involved and so it can be assumed the product of the reaction of H₂O₂ and Cu²⁺, the ‘caged’ OH', is the oxidising species.

Other studies of MCO of protein in autoimmune disease have lead to the generation of autoantigenic protein fragments or aggregates. Casciola-Rosen et al, 1997 found MCO was shown to specifically fragment autoantigens targeted in the disease Scleroderma. This, like SLE, is a systemic autoimmune disease characterised by the presence of a unique set of autoantibodies. The reversible ischemia-reperfusion that occurs specifically in this disease was suggested to underlie this oxidative effect which did not appear to occur in other systemic autoimmune diseases. In a different study by Trigwell et al, 2001 MCO was shown to generate autoantigenic aggregates in type I diabetes mellitus. In both studies the potential mechanisms of aggregation/fragmentation were studied for the importance in the understanding of how potential autoantigens are generated. For MCO of Ro60 in SLE, insensitivity to OH' scavenger and sensitivity to metal ion chelators strongly indicated metal binding to protein was required for oxidation. However a more definitive method used by both Casciola-Rosen et al, 1997 and Trigwell et al, 2001 was to address whether zinc (which is able to bind to the same site as Cu but is unable to support Fenton chemistry) might influence the ability of Cu to induced oxidative damage. Using both metal chelators and zinc the MCO induced fragmentation of autoantigens in Scleroderma was inhibited suggesting it to be a ‘caged’ process resulting in site specific damage, as was suggested for Ro60 aggregation. However, for the MCO generated autoantigenic aggregates in type I diabetes mellitus, in this study zinc which should compete for any metal chelation sites on the protein but not catalyse oxidative modification, was
unable to inhibit the aggregation and in this case the mechanism was suggested not to be dependent on site-specific interactions of the metal ions.

4.6.5 Protein oxidation in cell lysate

Ro60 oxidative damage using NDF cell lysate as a source of Ro60 was used to account for influences of the cellular environment on the oxidative process. Firstly, anti-Ro antibody from a commercially available anti-Ro/La positive serum was used to identify Ro60 in a lysate sample by Western blotting. Three bands were immunostained with one band being more prominent using commercial Ro positive serum containing antibodies to Ro and La. Three major autoantigens in SLE, which form part of the RoRNP complex, are Ro60, Ro52 and La and the molecular weights of these bands are 60kDa, 52kDa and 42kD respectively. These would be expected to be recognised using the anti-Ro/La serum and it is suggested that the observed three bands correspond to these proteins. Since the bands are close in molecular weight, purified Ro60 was run along side the lysate samples to help identify which band was Ro60. From the results of this it appeared that the less prominent band of the three ran to the same size as the purified Ro60, and this corresponded to a molecular weight of approximately 60kDa.

Identification of the Ro60 band based on the molecular weight of purified Ro60 overexpressed in E.coli may be different for Ro60 expressed in human cells. It is likely that human Ro60 is posttranslationally modified for example by phosphorylation or glycosylation and these modifications would not be present on Ro60 expressed in E.coli. These modifications have an effect on the apparent molecular weight of the protein. A good example of this is the BSE prion protein PrP which when resolved by SDS-PAGE resolves as a triplet which have been shown to represent un-glycosylated, mono and di-glycosylated forms of the protein which differ in molecular weight from each other by about 3kDa (J Owen, personal communication). The expression levels of the Ro proteins and La in cells have also been shown to differ, the number of La molecules has been estimated to be about 1 million and the number of Ro60 and Ro52 is believed to be 100 times smaller (Pruijn et al, 1997). We may therefore expect a prominent band to be represented by La. However the signal intensity of the bands will depend also on the avidity of the antibody to the protein as well as the amount of protein. Since identification of Ro60 was not clear it is more correct to suggest assessment of MCO induced oxidative changes of SLE autoantigens.

Western blotting with anti-Ro/La positive serum of an NDF lysate after MCO demonstrated the formation of a 25kDa immunoreactive fragment after treatment with the highest concentration of
H$_2$O$_2$/Cu$^{2+}$. This MCO-induced fragment was induced in a different system to the MCO of purified Ro60 and thus results cannot be compared. No immunoreactive aggregates were detected using this method but high molecular weight aggregates at the interface of the stacking and resolving gel and at the top of the stacking gel, using the carbonyl assay were detected. Results showed increasing levels of protein carbonyls with increased dose of H$_2$O$_2$ as was observed for purified Ro60. Under the assay conditions levels of carbonyls on the untreated protein were undetectable compared to the treated samples. The loss of low molecular weight carbonyl bands and the formation of the high molecular weight bands comparing the intermediate and high dose suggested that more than one protein may be forming part of the aggregate. Since many of the lysate protein may be susceptible to carbonyl formation it was not possible to say how many proteins and if Ro60 formed part of the carbonyl aggregate at the highest dose.

Fragmentation by MCO of several autoantigens has been seen in Scleroderma (a systemic autoimmune disease resulting in increased vasoreactivity and tissue fibrosis) however these results were not demonstrated in SLE and the reversible ischemia-reperfusion that occurs specifically in Scleroderma was suggested to underlie this oxidative effect (Casciola-Rosen et al, 1997). Goodpasture syndrome (a disease characterised by onset in mid-life of glomerulonephritis and pulmonary hemorrhage) is associated with autoantibodies to $\alpha$3 non-collagenous globular (NC1) domains. These are of type IV collagen forming part of a NC1 hexamer within the glomerular basement membrane (GBM). MCO of NC1 hexamer preparations in vitro demonstrated increased reactivity of Goodpasture autoantibody as well as protein structural changes resulting in first fragmentation followed by formation of high molecular weight aggregates. These effects were sensitive to inhibition by radical scavengers and metal ion chelators suggesting more than one species may be mediating the exposure. These cryptic epitopes are normally in a site of immune privilege buried within the GBM in vivo and ROS exposure of cryptic epitopes here has therefore been implicated in the pathogenesis of the disease (Kalluri et al, 2000).

4.6.6 Oxidation to Ro60 and implication for autoimmunity

In many autoimmune diseases including SLE the production of autoantibody is driven by antigen and is T cell dependent (Maran et al, 1993) and therefore involves breaking of T cell tolerance and the activation of autoreactive T cells. T cell tolerance is only maintained to dominant epitopes which are efficiently presented; altered protein conformation, novel cleavage or modification of protein structure may lead to presentation of cryptic epitopes to which T cells are not tolerant (Gammon and Sercarz, 1989). Under conditions of oxidative stress ROS have effects on the structure of proteins
representing a possible mechanism for revealing cryptic epitopes in autoimmune conditions (Casciola-Rosen et al., 1997; Kalluri et al., 2000). Ro60 may be a potential candidate for ROS induced modification in vivo where UVB may be a potential oxidising source for this protein (anti-Ro antibodies have been associated with photosensitivity in SLE (Mond et al., 1989) and this may be related to protein oxidation of Ro caused by exposure to UVB). Therefore UVB irradiation and MCO (a common ROS generating system) of Ro60 in vitro was investigated. Initially, global oxidation products were investigated, aggregation, fragmentation and carbonyl formation (Shacter, 2000). UVB did not cause oxidative changes to Ro60 under the experimental conditions applied, but MCO was able to induce both aggregation and carbonyl formation. Fragmentation of an SLE autoantigen was also observed after MCO using lysates from NDF cells.

The products of MCO of Ro60 detected using Western blotting and the carbonyl assay are an example of the many oxidative changes which may occur to Ro60 in vivo. The investigation of other oxidising sources for example HOCl (a potential oxidising species in inflammatory conditions as it is produced by activated neutrophils (Kettle and Winterbourne, 1997)) and other assays of protein oxidation may detect other protein oxidation products which may equally be involved in the activation of autoreactive T cells. There are many assays that could be employed with sufficient quantity of purified protein for a detailed investigation. A few examples are: the susceptible cysteine residues resulting in disulfide bond formation detectable by non-reducing SDS-PAGE (Davies and Delsignore, 1987), methionine sulfoxide detected by CNBr cleavage and amino acid analysis (Maier et al., 1995). The formation of protein hydroperoxides is also a favourable candidate as several amino acids are susceptible and can be detected by HPLC. Oxo-histidine, (Lewisch and Levine, 1995) another marker of UVB induced oxidation, could be investigated and dityrosine detection (Giulivi and Davies, 1994) could be used to further investigate the mechanism of MCO induced aggregate formation. The generation of different types and amount of oxidation damage related to the level of oxidative stress would also be important as this could be used to investigate the level and type of oxidative damage required for the activation of autoreactive T cells.

In the concluding chapter a method was established to detect T cell activation after antigen stimulation and this was used to investigate whether the product of MCO Ro60, ie in aggregated form containing increased levels of carbonyl groups, could activate autoreactive T cells. It is important to determine if these oxidative changes to protein autoantigen are able to break tolerance to self antigen and work done in this area is scarce. The activation of autoreactive T cells which recognise several fragments of topoisomerase I, an autoantigen in scleroderma, suggests that
fragmentation perhaps induced by ROS could be a possible mechanism (Kuwama et al, 1995). Also reactive aldehydes which are known to be generated during lipid peroxidation in cells have been used to modify homologous protein which broke T cell tolerance to self protein in immunised mice (Wuttage et al, 1999). However much of the evidence to support protein oxidation in the pathogenesis of disease is indirect, where particular pathologies have been associated with the accumulation of protein products indicative of oxidative damage. For example, oxidation induced aggregation of immunoglobulin molecules from the synovial fluid in rheumatoid arthritis has been suggested to be associated with the pathology of this disease. Another common observation is the deposition of aggregated protein consisting of dense fibrillar structures containing β-pleated sheet secondary structure that are found in many diverse neurodegenerative conditions (Kaytor and Warren, 1999).
CHAPTER 5:
CELL ACTIVATION AND DEATH RESPONSES OF SLE AND SJÖGREN'S SYNDROME PBMC TO PURIFIED AND ROS-MODIFIED RECOMBINANT Ro60
5.0 CELL ACTIVATION AND DEATH RESPONSE OF SLE AND SJÖGREN'S SYNDROME PBMC TO PURIFIED AND ROS-MODIFIED RECOMBINANT Ro60

5.1 Introduction

The autoantibody response to Ro60 has been characterised as a high affinity antibody of predominantly IgG1 subclass (Maran et al, 1993), this is a T cell dependent process where exogenous antigen uptake by antigen presenting cells (APC) such as dendritic cells, macrophages or B cells is processed into peptides that are carried to the cell surface by MHC class II molecules. These are presented to the cell surface by MHC class II molecules. These are presented to CD4 T helper cells via the MHC class II antigen-presenting pathway (Cresswell, 1994). No literature to date has been found on T cell epitopes of Ro60 despite the extensive study of the B cell epitopes of Ro60 (Wahren-Herlenius et al, 1999). The studies that have been carried out on T cell epitopes to other SLE autoantigens are also limited, one study investigated MHC Class II-bound self peptides from autoimmune MRL/lpr mice and these were isolated and sequenced and compared to those from similar preparations of non-autoimmune mice. Those that did not appear in controls appeared to represent components of SLE autoantigens and were suggested to represent potential SLE T cell epitopes (Freed et al, 2000).

The production of cytokines which induce or mediate most immune responses is a good starting point for the analysis of T cells which are activated in an immune response. Of particular interest is the identification of groups and subgroups of T cells activated during an immune response and if these are altered in certain autoimmune disease states. The secretion of the effector cytokines, IFNγ and IL-4 can be used to investigate activation of primed antigen specific effector or memory immune cells following short in vitro stimulation of PBMC with specific antigen. The autoimmune response to Ro60 in SLE is expected to activate a T helper cell response as these are required for the production of the high affinity antibody to Ro60, which has already been characterised (Maran et al, 1993). PBMC from SLE subjects who are positive for the presence of Ro60 antibody should contain memory and effector T cells primed against the Ro60 autoantigen.

It is suggested that in vitro stimulation with recombinant Ro60 or ROS damaged Ro60 may cause these cells to become activated by either antigen depending on whether the autoantibody response in SLE subjects is triggered by native or oxidised Ro60; with activation resulting in the production of IFNγ and/or IL-4. A positive cytokine response to these antigens would not be expected using cells
from normal healthy controls as their cells should not have been primed against this self protein and these could be used a control group for the identification of an autoimmune response to Ro60 \textit{in vitro} in SLE.

UV has been shown to induce apoptosis in human keratinocyte cell lines from photosensitive SLE patients resulting in autoantigens being clustered in two populations of surface structures on apoptotic keratinocytes (Casciola-Rosen \textit{et al}, 1994). Apoptosis is a highly organised form of cell death essential for maintaining homeostatic function in a variety of tissues and is characterised by cellular shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing and the generation of apoptotic vesicles (Kerr \textit{et al}, 1972). Apoptotic vesicles are removed by the phagocytic system to avoid major inflammatory reactions (Fadock \textit{et al}, 1993). The two populations of apoptotic vesicles induced in human keratinocyte cell lines from photosensitive SLE patients contain clusters of autoantigens from the ER and nuclear membranes, which are sites of ROS generation in apoptotic cells and oxidative damage at these sites may also lead to modification of ‘self’ antigens uniting these molecules as autoantigens (Casciola-Rosen \textit{et al}, 1994). The regulation of apoptosis may play a role in autoimmunity, if the apoptotic cell is the supplier of autoantigens. In this case too much cell death may prevent efficient removal by the phagocytic system and autoantigens present in surface blebs could be a target for other ROS modifications. Alternatively too little cell death may also have implications for autoimmunity, for example, by insufficient removal of autoreactive clones by defective T cell apoptosis in the thymus during the development of T cell tolerance (Greidinger, 2001).

In addition to the study of immune cell activation by autoantigen in SLE the process of cell death was also be investigated, as cell death, in particular apoptosis, plays an important role in many aspects of immune function including the response to antigen and it is possible that impairment of this process may be a factor in the development an autoimmune response. The percentage of apoptotic and necrotic cells was therefore considered as well as the activation of immune cells when investigating the immune response to autoantigen \textit{in vitro} in peripheral lymphocytes from SLE subjects. The investigation of cell death responses as well as activation responses will reflect the overall immune response to antigen as a combination of both processes will influence the final response.
5.2 Aims

The overall aim was to investigate if modification of Ro60 by ROS could play a role in the development of an autoimmune response against this autoantigen.

The aim of the first section (section 5.5) was to monitor the immune response to antigen in vitro by antigen stimulation of PBMC and the detection of secreted cytokines following activation of specific cells. To achieve this the parameters were optimised for the detection of cytokine secreting cells by flow cytometry using the MACS cytokine secretion assay kits with a positive control. This data was used to investigate effects of cellular responses of SLE PBMC stimulated non-specifically compared to the control samples. Three separate concentrations of recombinant Ro60 were used to investigate if PBMC from SLE, SS or normal subjects responded to in vitro stimulation with this protein. Finally, the in vitro response to ROS damaged Ro60 was monitored using PBMC from SLE or SS subjects compared to normal control subjects.

In the second section (section 5.6) the aim was to investigate cell death responses by measurement of apoptotic, necrotic and viable PBMC from Ro positive subjects with SLE or SS compared to those of normal controls with and without stimulation with different antigens. Using flow cytometry, a standard method for distinguishing apoptotic, necrotic and viable cells by dual staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide was applied. Firstly percentages of apoptotic, necrotic and viable peripheral lymphocytes in both groups of volunteers after in vitro incubation without any antigen stimulation were compared. Secondly, cell death responses were compared after non-specific stimulation in vitro by incubation with SEB; and specific stimulation with recombinant Ro60 and Ro60 which had been oxidatively modified by ROS.
5.3 Methods
The MACS cytokine secretion assay was used to investigate the autoimmune response to Ro60 in SLE cells in vitro. Cytokine secreting cells were analysed by flow cytometry. This technique enabled the detection of individual live cytokine secreting cells following stimulation with Ro60 or modified Ro60. This method is very sensitive and should provide more reliable information on the distribution of cytokines measured than methods reflecting the cytokine production of whole cell populations. Assaying at the single cell level may help in the understanding of disease immunopathology as potentially live antigen specific T cells could be isolated for analysis of T cell receptor repertoire and the mapping of T cell receptor epitopes.

5.3.1 Study subjects
Patients with a positive serological test for the presence of anti-Ro60 antibody, including patients with Sjögren's syndrome (SS) and mixed connective tissue disease (MCTD) as well as SLE were recruited from Dr Hassan's Rheumatology clinic at the Leicester Royal Infirmary by Dr Alison Kinder, Clinical Registrar. Age- and sex-matched controls were obtained from healthy laboratory and secretarial personal working in the University of Leicester, Department of Pathology. The study was approved by the Leicestershire Health Authority Research Ethics Committee and informed written consent was obtained before the collection of blood samples. Individual patient and control data including age, sex, and disease phenotype; disease activity at the time of taking blood, immunology test results and current treatment are given in table 5.1 A-E.

5.3.2 Isolation of PBMC and treatment with antigen in vitro
PBMC were isolated from blood samples by density centrifugation as described in section 2.6.1. Antigens SEB, Ro60 and ROS damaged Ro60 were incubated with PBMC as described in section 2.6.2 and cells with no antigen were included as controls.

5.3.3 Detection of IFNγ and IL-4 secreting cells
MACS cytokine secretion assay kits were used to label of IFNγ and IL-4 secreting cells (section 2.6.3) following stimulation of PBMC with antigen in vitro and the cytokine secreting positive cells were then detected by flow cytometry (section 2.6.4). Two approaches were applied for the detection of cytokine responses using dual fluorescent labelling. The first approach was to label CD4 and IFNγ secreting cells based on the theory that CD4 cells would be activated as these would be required for the production of the high affinity antibody to Ro60 and because considerable evidence suggested that IFNγ may play a role in SLE immunopathology (Fan and Wuthrich, 1997; Seery et al, 1997; al-
Janadi et al., 1993). The second approach was to label all IFNγ and IL-4 secreting cells in the leukocyte population as the cells and cytokines involved in the immune response to Ro60 in SLE have yet to be identified.

5.3.4 Measurement of cell death
Flow cytometry was used to investigate the effect of antigen treatment on cell death in vitro of lymphocytes obtained from Ro positive and control subjects. Apoptotic, necrotic and viable cells were distinguished by dual staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and Propidium Iodide as described in section 2.6.5.
<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>Main Diagnosis (other problems)</th>
<th>Immunology</th>
<th>Treatment</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>73</td>
<td>F</td>
<td>Primary SS (hypertension, glaucoma, osteoarthritis)</td>
<td>Ro &amp; La +ve</td>
<td>-</td>
<td>Atenolol, Aspirin, Thyroxine sodium, Bendrofluazide, Eye drops, Co-codamol, Cimetidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ANA -ve</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DNA 0-30 IU/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C3 (0.85-1.98g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C4 (0.14-0.54g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>46</td>
<td>F</td>
<td>MCTD (alopecia, Raynaud's, Scleroderma skin thickening, shortness of breath, difficulty in swallowing)</td>
<td>Ro &amp; La +ve</td>
<td>*C3 0.83? *C4 0.09?</td>
<td>Amitriptyline, Amlodipine (winter)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ANA 1:256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>29</td>
<td>F</td>
<td>SLE</td>
<td>Ro &amp; La +ve</td>
<td>*DNA 120</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ANA +ve</td>
<td>*C3 1.05? *C4 0.09?</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 5.1 A Individual Ro positive subjects (R1-R3) data for subjects who gave blood samples for the study. Data include age, sex, main diagnosis, relevant immunology blood test results (Ro/La, ANA, dsDNA and complement activity), current treatment and assessment of disease activity at time of giving blood. Immunology results were based on results of blood tests performed on samples taken nearest to the time of taking blood for the study. Disease activity was assessed on notes made following clinical examination of the patient by the clinician at the clinic appointment nearest to the time of taking blood for the study. Also considered were the results of dsDNA and C3 and C4 complement activity where raised dsDNA levels and reduced C3 and C4 complement are markers of active disease. * indicates that the dsDNA and C3 and C4 complement levels are outside of the normal range. For C3 and C4 complement levels a ? indicates that in vitro activation cannot be excluded as a cause of low complement levels as the sample was received by the lab one day after collection.
<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>Main Diagnosis (other problems)</th>
<th>Immunology</th>
<th>Treatment</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ro/La +ve DNA &gt;300 C3 1.023 C4 0.28</td>
<td>hospital patient active</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lansaprazole Tramadol Amirtrityline Prednisolone Azathioprine Cyclosporin Alendronate Fluconazole Clarithromycin Paracetamol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R5 49 F SLE (autoimmune liver disease) Ro +ve ANA 1:64 DNA 2 C3 1.21 C4 0.16</td>
<td>app to give blood for the study inactive (but photosensitive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Azathioprine Thyroxine sodium Hydroxychloroquine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R6 51 F SLE (asthmatic) Ro +ve ANA -ve *DNA 33 C3 1.04 C4 0.21</td>
<td>regular clinic app inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hydroxychloroquine Prednisolone HRT Co-proxamol</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 B Individual Ro positive subjects (R4-R6) data for subjects who gave blood samples for the study. Data include age, sex, main diagnosis, relevant immunology blood test results (Ro/La, ANA, dsDNA and complement activity), current treatment and assessment of disease activity at time of giving blood. Immunology results were based on results of blood tests performed on samples taken nearest to the time of taking blood for the study. Disease activity was assessed on notes made following clinical examination of the subjects by the clinician at the clinic appointment nearest to the time of taking blood for the study. Also considered were the results of dsDNA and C3 and C4 complement activity where raised dsDNA levels and reduced C3 and C4 complement are markers of active disease. * indicates that the dsDNA and C3 and C4 complement levels are outside of the normal range.
Table 5.1 C Individual Ro positive subjects (R7-R10) data for subjects who gave blood samples for the study. Data include age, sex, main diagnosis, relevant immunology blood test results (Ro/La, ANA, dsDNA and complement activity), current treatment and assessment of disease activity at time of giving blood. Immunology results were based on results of blood tests performed on samples taken nearest to the time of taking blood for the study. Disease activity was assessed on notes made following clinical examination of the patient by the clinician at the clinic appointment nearest to the time of taking blood for the study. Also considered were the results of dsDNA and C3 and C4 complement activity where raised dsDNA levels and reduced C3 and C4 complement are markers of active disease. * indicates that the dsDNA and C3 and C4 complement levels are outside of the normal range. For C3 and C4 complement levels, a question mark indicates that in vitro activation cannot be excluded as a cause of low complement levels as the sample was received by the lab one day after collection.

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>Main Diagnosis (other problems)</th>
<th>Immunology</th>
<th>Treatment</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C3 (0.85-1.98 g/L)</td>
<td>Methotrexate, Folic Acid, Prednisolone, Nifedipine</td>
</tr>
<tr>
<td>R7</td>
<td>31</td>
<td>F</td>
<td>SLE (systemic vasculitis Raynauds)</td>
<td>Ro +ve ANA 1:256 DNA 0.30 IU/ml</td>
<td>*DNA 177</td>
<td>*C3 0.72? *C4 0.10?</td>
</tr>
<tr>
<td>R8</td>
<td>28</td>
<td>F</td>
<td>SLE</td>
<td>Ro +ve ANA -ve</td>
<td>*DNA 36</td>
<td>*C3 0.72 *C4 0.1</td>
</tr>
<tr>
<td>R9</td>
<td>65</td>
<td>F</td>
<td>Primary SS</td>
<td>Ro &amp;La +ve ANA -ve</td>
<td>-</td>
<td>C3 1.04 C4 0.24</td>
</tr>
<tr>
<td>R10</td>
<td>22</td>
<td>F</td>
<td>Cutaneous LE</td>
<td>Ro &amp;La +ve</td>
<td>-</td>
<td>C3 1.07 C4 0.19</td>
</tr>
</tbody>
</table>

For C3 and C4 complement levels, a question mark indicates that in vitro activation cannot be excluded as a cause of low complement levels as the sample was received by the lab one day after collection.
Table 5.1 D Individual Ro positive subjects (R7-R10) data for subjects who gave blood samples for the study. Data include age, sex, main diagnosis, relevant immunology blood test results (Ro/La, ANA, dsDNA and complement activity), current treatment and assessment of disease activity at time of giving blood. Immunology results were based on results of blood tests performed on samples taken nearest to the time of taking blood for the study. Disease activity was assessed on notes made following clinical examination of the patient by the clinician at the clinic appointment nearest to the time of taking blood for the study. Also considered were the results of dsDNA and C3 and C4 complement activity where raised dsDNA levels and reduced C3 and C4 complement are markers of active disease. * indicates that the dsDNA and C3 and C4 complement levels are outside of the normal range.
<table>
<thead>
<tr>
<th>Control Subjects</th>
<th>Main Diagnosis (other problems)</th>
<th>Immunology</th>
<th>Markers of activity</th>
<th>Treatment</th>
<th>Disease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>Age</td>
<td>Sex</td>
<td>ENA &amp; ANA</td>
<td>Ro/La +ve</td>
<td>ANA (IgG titre)</td>
</tr>
<tr>
<td>C1</td>
<td>28</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>33</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>44</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>28</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>24</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>68</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>44</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C9</td>
<td>24</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>44</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>41</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12</td>
<td>80</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 E Individual control subjects (C1-C12) data for healthy volunteers who were recruited on the basis of age and sex to match with the Ro positive subjects. In the case where Ro positive subjects could not be matched for age by healthy laboratory or secretarial staff working in the University of Leicester, Department of Pathology; hospital in-patient volunteers who were negative for the presence of autoantibody to Ro were invited to donate a blood sample for the study. Data include age, sex, main diagnosis and current treatment where appropriate.
5.4 Results

5.4.1 Age and sex matched Ro positive and control subjects

Age and sex of matched Ro positive and control subjects Table 5.2. This shows that the subjects were matched for age and sex. All but one of the Ro60 positive patients were female with a mean age of 48 ± 23 years. Female volunteers were therefore enrolled into the control group except for one male subject.

<table>
<thead>
<tr>
<th>Ro positive subject</th>
<th>Sex</th>
<th>Age</th>
<th>PBMC per ml of blood</th>
<th>Control Subject</th>
<th>Sex</th>
<th>Age</th>
<th>PBMC per ml of blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>F</td>
<td>29</td>
<td>1.10 x 10^6</td>
<td>C1</td>
<td>F</td>
<td>28</td>
<td>0.50 x 10^6</td>
</tr>
<tr>
<td>R4</td>
<td>M</td>
<td>32</td>
<td>0.10 x 10^6</td>
<td>C2</td>
<td>M</td>
<td>33</td>
<td>0.70 x 10^6</td>
</tr>
<tr>
<td>R5</td>
<td>F</td>
<td>49</td>
<td>0.55 x 10^6</td>
<td>C3</td>
<td>F</td>
<td>44</td>
<td>0.46 x 10^6</td>
</tr>
<tr>
<td>R7</td>
<td>F</td>
<td>31</td>
<td>0.37 x 10^6</td>
<td>C5</td>
<td>F</td>
<td>28</td>
<td>0.50 x 10^6</td>
</tr>
<tr>
<td>R8</td>
<td>F</td>
<td>28</td>
<td>0.50 x 10^6</td>
<td>C6</td>
<td>F</td>
<td>24</td>
<td>1.10 x 10^6</td>
</tr>
<tr>
<td>R9</td>
<td>F</td>
<td>65</td>
<td>0.85 x 10^6</td>
<td>C7</td>
<td>F</td>
<td>68</td>
<td>0.11 x 10^6</td>
</tr>
<tr>
<td>R5</td>
<td>F</td>
<td>49</td>
<td>0.55 x 10^6</td>
<td>C8</td>
<td>F</td>
<td>44</td>
<td>0.41 x 10^6</td>
</tr>
<tr>
<td>R10</td>
<td>F</td>
<td>22</td>
<td>0.73 x 10^6</td>
<td>C9</td>
<td>F</td>
<td>24</td>
<td>1.10 x 10^6</td>
</tr>
<tr>
<td>R11</td>
<td>F</td>
<td>40</td>
<td>0.41 x 10^6</td>
<td>C10</td>
<td>F</td>
<td>44</td>
<td>0.41 x 10^6</td>
</tr>
<tr>
<td>R12</td>
<td>F</td>
<td>37</td>
<td>1.50 x 10^6</td>
<td>C11</td>
<td>F</td>
<td>41</td>
<td>1.10 x 10^6</td>
</tr>
<tr>
<td>R13</td>
<td>F</td>
<td>85</td>
<td>0.23 x 10^6</td>
<td>C12</td>
<td>F</td>
<td>80</td>
<td>0.45 x 10^6</td>
</tr>
</tbody>
</table>

Table 5.2: Ro positive subjects were age and sex matched with control subjects. The individual matched subjects and controls are shown and the average age ± STDEV and range in each groups is given. Yield of PBMC are also given, cell number per ml of blood was calculated by counting PBMC in 5 of 25 squares within the triple-line markings in a haemocytometer grid and calculated by multiplying the number of cells in 25 squares by the dilution factor and by a factor of 10^6.
5.4.2 Yield of PBMC

PBMC were isolated from blood samples by Histopaque gradient separation and the number of PBMC cells in 1ml of blood was counted (Table 5.2). The mean number of PBMC was not different in the Ro positive group, 6.3±4.0×10⁵ cells per ml of blood compared with the control group, 6.2±3.4×10⁵ cells per ml of blood. The cells were assessed as 100% viable in both groups by staining with Trypan blue solution.

5.4.3 Preparation of Ro60 and ROS Ro60 for in vitro stimulation of PBMC

Purified Ro60 was produced as described in chapter 3 and was stored in aliquots at -80°C ready to use for the stimulation of PBMC. Western blotting with anti-Ro antibody was carried out using serum from a Ro positive subject recruited to the study. This showed the purified Ro60 could be identified using a non-commercial Ro positive serum (Figure 5.1: i) B). ROS damaged Ro60 also to be used in the stimulation of PBMC was prepared in chapter 4 and was stored in aliquots at -80°C. Freezing was shown to have no apparent effect on the ROS damaged Ro60, Figure 5.1: ii) C carbonyl groups present on the high molecular weight aggregate following MCO were still present following storage. The antigens for cell stimulation work were prepared in a class II cabinet using sterile practice, the purified Ro60 was filter sterilised as an extra precaution. A new batch of ROS damaged Ro60 was prepared using filter sterilised Ro60 treated with filter sterilised H₂O₂ and Cu and stored in aliquots at -80°C. This was tested for oxidative damage prior to starting the in vitro stimulation work to show that Ro60 did not interact with the membrane during filter sterilisation (Figure 5.1: ii) F). No carbonyls or aggregate was detected on non-treated Ro60 (Figure 5.1: ii) E).

![Western blot immunostaining using serum from a Ro positive subject diluted 1:250 for the detection of purified Ro60 (i). Detection of protein carbonyls on an aggregate of Ro60 produced following MCO of Ro60 using anti-DNP antibody (ii). A: molecular weight markers. B: purified Ro60. C: MCO treated Ro60 which had been stored at -80°C. E: Non treated filter sterilised Ro60. F MCO treated filter sterilised Ro60.](image-url)
5.4.4 Flow cytometric analysis of PBMC was used detect a live lymphocyte population

Analysis of light forward scatter and side scatter was used to identify a live lymphocyte population based on size and granularity (Figure 5.2). This was used to set a gate for the analysis of IFN γ (CD4+ and CD4-) and IL-4 secreting cells; and in the second part of the chapter annexin V and PI positive lymphocytes.

![Diagram of dot-plot showing forward versus side scatter of PBMC used for setting a live lymphocyte gate.](image)

Figure 5.2: Dot-plot of forward versus side scatter of PBMC used for setting a live lymphocyte gate. Light scatter was used to gate lymphocytes within the PBMC population so that fluorescence data would only be collected from these cells. A PBMC sample was passed through the laser beam of the flow cytometer where the light scatter is changed based on the size (forward scatter) and granularity (side scatter) of each cell. This enabled the small granular lymphocytes to be differentiated from large and very granular cells, dead cells, cell aggregates and cell debris.
5.5 THE EFFECT OF Ro60 AND ROS-MODIFIED Ro60 ON SLE AND SS PBMC CYTOKINE SECRETION.

5.5.1 Flow cytometric analysis of IFNγ (CD4+ and CD4-) and IL-4 secreting cells

Data on the percentage of IFNγ (CD4+ and CD4-) and IL-4 secreting cells were collected using flow cytometry, collecting 10,000 events. Typical 2-colour dot plots i) IFNγ versus CD4 and ii) IFNγ versus IL-4 from a healthy control subject whose lymphocytes had been stimulated with SEB are shown in Figure 5.3. These results illustrate the detection of the following cells i) IFNγ secreting CD4- (A1), IFNγ secreting CD4+ (A2), CD4+ (A4) and non IFNγ secreting CD4- (A3). ii) IL-4 secreting (A1), IFNγ and IL-4 secreting (A2), IFNγ (A4) and non IFNγ and IL-4 secreting (A3) (n=10,000 events).

Figure 5.3i PBMC isolated from a healthy volunteer was used to detect IFNγ+ (CD4- and CD4+) lymphocytes after stimulation with SEB for 6 hrs by flow cytometry. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of CD4+ cells. The 2-colour dot plot shows the fluorescence data for IFNγ-PE (emission 578nm detected in channel 2) and CD4-FITC (emission 520nm detected in channel 1) acquiring 10,000 events set with a live lymphocyte gate. Quadrant A3 of the dot plot reflects non-IFNγ secreting CD4- cells. Quadrant A4 shows non-IFNγ secreting CD4+ cells. IFNγ secreting CD4+ cells are shown in quadrant A2 and IFNγ secreting CD4- in quadrant A1.
Figure 5.3ii PBMC isolated from a healthy volunteer was used to detect IFNγ+ and/or IL-4 secreting cells after stimulation with SEB for 6 hrs by flow cytometry. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ and an IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IFNγ and IL-4 was detected by anti-IFNγ-FITC and IL-4-PE. The 2-colour dot plot shows the fluorescence data for IL-4-PE (emission 578nm detected in channel 2) and IFNγ-FITC (emission 520nm detected in channel 1) acquiring 10,000 events set with a live lymphocyte gate. Quadrant A3 of the dot plot reflects non-IL-4 or IFNγ secreting cells. Quadrant A4 shows IFNγ secreting cells and IL-4 secreting cells are shown in quadrant A1. Cells secreting both IFNγ and IL-4 are shown in quadrant A2.

5.5.2 PMA/ionomycin and SEB positive controls for the detection of IFNγ secreting, CD4+ cells

PBMC were isolated from two Ro (& La) positive subjects (R1 and R2). The subjects were diagnosed as having SS (R1) and MCTD (R2); the subjects were not any immunosuppressive treatment (Table 5.1A). The PBMC were stimulated non-specifically in vitro with PMA/Ionomycin and Staphylococcal Enterotoxin B (SEB). The percentage of IFNγ secreting cells was detected after stimulation in a 45 minute secretion period by using the MACs IFNγ secretion assay detection kit with flow cytometric analysis. The cells were also counterstained for the detection of CD4 positive cells using an anti-CD4 antibody, also detected by flow cytometry. Figure 5.4 shows the percentage of IFNγ secreting cells including the CD4 IFNγ secreting cells before and after antigen stimulation with PMA/I (Figure 5.4 A) and SEB (Figure 5.4 B). The percentage of total CD4 cells in the sample population is also shown before and after antigen stimulation in each figure. The percentage and
number of cells are also shown in Table 5.3. The data demonstrated that SEB could be used as a positive control for the detection of IFNγ secreting cells in Ro positive subjects, in this case resulting in 10% of the cells secreting IFNγ after stimulation. The percentage of total CD4 positive cells detected was approximately 45% of the peripheral blood lymphocytes, consistent with the percentage value for a normal subject population (BD Bioscience, technical information). The use of PMA/I as a positive control in this assay was not recommended in the manufacturers guide, as it is a strong stimulator resulting in high frequencies of IFNγ secreting cells and this does not enable conclusions on the sensitivity of the assay to be drawn. The data showed strong stimulation of the cells (63%) as expected by PMA/I. This stimulus is also known to cause the down regulation of the CD4 receptor on CD4 positive cells, which was confirmed here as the percentage of total CD4 positive was reduced from 39% to just 3% after stimulation (Figure 5.4 R1). To conclude, SEB was adopted for use as a positive control in this assay.

Figure 5.4 Total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes isolated from two Ro positive subjects after antigen stimulation with PMA/I (R1) and SEB (R2) for 16 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the % (and number) of total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes after antigen stimulation compared to unstimulated cells.
Table 5.3 Total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes isolated from two Ro positive subjects after antigen stimulation with PMA/I (R1) and SEB (R2) for 16 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % of total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes after antigen stimulation (S). These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

### 5.5.3 Detection of CD4+ IFNγ secreting cells in response to Ro60

PBMC were isolated from three Ro positive subjects and three age matched controls. For each subject a cell sample was stimulated with antigen by incubation with 10µg/ml of Ro60 for 16hr. The antigen concentration and incubation time (the maximum incubation time suggested for protein antigen) was recommended in the IFNγ secreting assay protocol from the manufacturer. Cell samples were also incubated with 10µg/ml SEB (+ve control) and no antigen, used as a negative control. The percentage of CD4+ and CD4- IFNγ secreting cells was determined after stimulation in a 45 minute secretion period in a 10ml volume by using the MACs IFNγ secretion assay detection kit with flow cytometric analysis.

The analysis of the first two age- and sex-matched subjects showed the percentage of CD4+ and CD4- IFNγ secreting responses to Ro60 were less than 5% and so the secretion volume was reduced to 1ml for the third subject to improve the sensitivity of the assay. The percentage and number of CD4+ and CD4- IFNγ secreting cells after stimulation with SEB and Ro60 are given in Table 5.4. For each subject and age matched controls the fold change from control (no antigen) was calculated from the percentages of CD4+ and CD4- IFNγ secreting cells after stimulation with SEB and Ro60 and are shown in Figure 5.5. Control cells without the addition of either detection antibodies to correct for background fluorescence were not measured in two of the six subjects. Therefore for consistency in this experiment, results were not corrected for background fluorescence in any of these subjects.

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Table 5.3 Total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes isolated from two Ro positive subjects after antigen stimulation with PMA/I (R1) and SEB (R2) for 16 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % of total IFNγ+ (CD4- and CD4+) and CD4+ secreting lymphocytes after antigen stimulation (S). These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

### 5.5.3 Detection of CD4+ IFNγ secreting cells in response to Ro60

PBMC were isolated from three Ro positive subjects and three age matched controls. For each subject a cell sample was stimulated with antigen by incubation with 10µg/ml of Ro60 for 16hr. The antigen concentration and incubation time (the maximum incubation time suggested for protein antigen) was recommended in the IFNγ secreting assay protocol from the manufacturer. Cell samples were also incubated with 10µg/ml SEB (+ve control) and no antigen, used as a negative control. The percentage of CD4+ and CD4- IFNγ secreting cells was determined after stimulation in a 45 minute secretion period in a 10ml volume by using the MACs IFNγ secretion assay detection kit with flow cytometric analysis.

The analysis of the first two age- and sex-matched subjects showed the percentage of CD4+ and CD4- IFNγ secreting responses to Ro60 were less than 5% and so the secretion volume was reduced to 1ml for the third subject to improve the sensitivity of the assay. The percentage and number of CD4+ and CD4- IFNγ secreting cells after stimulation with SEB and Ro60 are given in Table 5.4. For each subject and age matched controls the fold change from control (no antigen) was calculated from the percentages of CD4+ and CD4- IFNγ secreting cells after stimulation with SEB and Ro60 and are shown in Figure 5.5. Control cells without the addition of either detection antibodies to correct for background fluorescence were not measured in two of the six subjects. Therefore for consistency in this experiment, results were not corrected for background fluorescence in any of these subjects.

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Table 5.4 IFNγ+ CD4- and IFNγ+ CD4- secreting lymphocytes isolated from three Ro positive subjects (R3, R4 and R5) and age and sex-matched controls (C1, C2 and C3) after antigen stimulation with SEB and Ro60 10μg/ml for 16 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ CD4- and IFNγ+ CD4-secreting lymphocytes after stimulation with SEB and Ro60. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

The results showed that after stimulation with SEB all the subjects had IFNγ+, CD4- and four of six IFNγ+, CD4+ levels greater than the no antigen control but there was no difference between the two groups. None of the Ro positive or control subjects had an IFNγ+, CD4- or IFNγ+, CD4+ levels greater than the control after stimulation with Ro60. In conclusion both Ro positive subjects and control subjects had higher levels of IFNγ secretion after SEB treatment but an overall response to Ro60 was not observed in either group. All the Ro positive subjects were diagnosed with SLE. Two were being treated with immunosuppressive drugs (R4 and R5) and one was not receiving any treatment (R3). One subject had active disease (R5). The cytokine response to SEB was not greater in the subject not being treated (R3) compared to those on immunosuppressant (R4 and R5). The cytokine response to SEB was also not greater in the subject (R4) with active disease (being treated with immunosuppressant) compared to the subject R5, also on immunosuppressant but with non-active disease. The levels of IFNγ were increased following stimulation with SEB in subjects (R5 and C5) where a 1ml secretion volume was used and this may have increased the sensitivity of the assay.
Figure 5.5 IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes isolated from three Ro positive subjects (R3, R4 and R5) and age and sex-matched controls (C1, C2 and C3) after antigen stimulation with SEB and Ro60 10μg/ml for 16 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was labelled using anti-IFNγ-PE. Cells were also labelled with anti-CD4-FITC for the detection of IFNγ+ , CD4+ cells. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
5.5.4 Detection of IFN\(\gamma\) and IL-4 secretion from cells in response to stimulation with Ro60

As there was no IFN\(\gamma\) production in either CD4- or CD4+ cells to Ro60 under the experimental conditions described above it was therefore decided to continue to measure IFN\(\gamma\) (CD4- and CD4+ combined) but also IL-4 secreting cells following stimulation with Ro60. In addition, Ro60 concentrations of 5, 10 and 20\(\mu g/ml\) were used as 10\(\mu g/ml\) may not be the optimum concentration for antigen stimulation with this protein. The percentages of IFN\(\gamma\) secreting and IL-4+ cells were detected after stimulation in a 45 minute secretion period in a 5ml volume by using the MACs IFN\(\gamma\) secretion assay detection kit in combination with the IL-4 secretion assay detection kit with flow cytometric analysis.

Comparison of dual and single cytokine secreting cell detection

To test if the assay kits would work effectively in combination, PBMC were isolated from a Ro positive subject (R6) and stimulated with SEB during 4hr incubation as demonstrated in the IL-4 kit instructions to show an IL-4+ response. IL-4 and IFN\(\gamma\) were detected using the cytokine detection antibodies separately and in combination. The results in Figure 5.6 show that after SEB treatment IFN\(\gamma\) production was increased 5 fold when measured separately and 6.7 fold in combination with the IL-4 antibody compared to the non-treated control. However, the 2.3 fold increase of IL-4 to SEB was found to be less than the non-stimulated control when this cytokine was measured in combination with IFN\(\gamma\). In a second experiment (Ro positive subject R7) using the dual cytokine assay stimulating cells with SEB and Ro60 an IL-4+ increase (Figure 5.7) was demonstrated after stimulation with Ro60 which increased with increasing protein concentration. It was therefore decided to continue using this dual detection assay to investigate this observation further on a two more Ro positive subjects. Both subjects R6 and R7 were Ro +ve SLE and both being treated with immunosuppressants. Disease activity in SLE may be a factor effecting in vitro response to stimulation with Ro60 and might explain the differing responses to Ro60. However in these cases this could not be compared between the two subjects because although subject R6 had non-active disease for subject R7 the blood sample was not taken at a regular clinic appointment and disease activity at the time of taking blood was not known. The last immunology test result reported very high DNA levels and low complement C3 & C4 levels, indicators of active disease but this a was 8 months prior to giving blood and was not an indication of disease activity at the time of taking blood.
Table 5.5 IFNγ+ and IL-4+ (CD4- and CD4+) secreting lymphocytes isolated from a Ro positive subject (R6) after antigen stimulation with SEB 4 hours. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ and an IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IFNγ and IL-4 was the detected by anti-IFNγ-FITC and IL-4-PE. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ and IL-4+ secreting lymphocytes after stimulation with SEB. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence. To investigate if both kits could be used in combination on the same sample a comparison using just one detection antibody either IFNγ or IL-4 and also both antibodies together was made.

| Subject | Total IFNγ (CD4- and CD4+) | Total IL-4 (CD4- and CD4+) |  |
|---------|-----------------------------|-----------------------------|  |
|         | Dual cytokine antibody      | Single cytokine antibody    |  |
|         | Blk | U  | SEB | Blk | U  | SEB | Blk | U  | SEB | Blk | U  | SEB |  |
| R6      | 2  | (0.14) | 35 | (0.45) | 71 | (2.23) | 24 | (1.68) | 3  | (0.22) | 12 | (0.15) | 20 | (0.51) | 6  | (0.19) |

Figure 5.6 IFNγ+ and IL-4+ secreting lymphocytes isolated from a Ro positive subject (R6) after antigen stimulation with SEB 4 hours. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ and an IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IFNγ and IL-4 was the detected by anti-IFNγ-FITC and IL-4-PE. To investigate if both kits could be used in combination on the same sample a comparison using a single detection antibody (either IFNγ or IL-4) and both detection antibodies was made. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
Measurement of IFNγ and IL-4, 6 and 16hr after the onset of Ro60 stimulation

Upon stimulation with protein, the cells may be analysed for cytokine secretion within approximately 6-16hr after onset of stimulation. Initial measurements of IFNγ secretion were determined 16hr after stimulation with Ro60. Since no IFNγ production was detected this may have been because the stimulation period was too long. The period of incubation with antigen before cytokine secretion is stimulated may also be different for the release of different cytokines. To test this, PBMC isolated from a Ro positive subject (R7) were stimulated with SEB and 5, 10 and 20μg/ml of Ro60 for 6hr and 16hr followed by the detection of IFNγ and IL-4 using dual cytokine labelling secretion kits and analysed by flow cytometry. The fold changes in cytokine secretion after stimulation compared to the non-stimulated controls are shown in figure 5.7 (number and % of cytokine secreting cells for this experiment is shown in Table 5.6). The results show IFNγ secretion after stimulation with SEB for 16hr to be higher than after 6hr. The IL-4 secretion after SEB stimulation was not increased compared to the control cells following 6 or 16hr incubation. The cells showed an IL-4 increase after stimulation with Ro60 at 10 and 20μg/ml after 6hr incubation with antigen but a similar trend was not observed at 16hr. It was therefore decided to measure IFNγ and IL-4, under appropriate conditions, in two further Ro positive subjects and match the subjects with controls.

Figure 5.7: IFNγ, IL-4 and IFNγ & IL-4 secreting lymphocytes isolated from a Ro positive subject (R7) after antigen stimulation with SEB and 3 concentrations of Ro60 (5μg/ml, 10μg/ml 20μg/ml) following 6 (A) and 16 (B) hours incubation were investigated. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits in combination. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ and an IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IFNγ and IL-4 was detected by anti-IFNγ-FITC and and IL-4-PE. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
Table 5.6 IFN\(_\gamma\), IL-4 and IFN\(_\gamma\) & IL-4 secreting lymphocytes isolated from a Ro positive subject (R7) after antigen stimulation with SEB and 3 concentrations of Ro60 (5\(\mu\)g/ml, 10\(\mu\)g/ml 20\(\mu\)g/ml) following 6 and 16 hours incubation were investigated. IFN\(_\gamma^+\) and IL-4 secreting cells were labelled using the MACs IFN\(_\gamma\) and IL-4 secretion assay kits. An IFN\(_\gamma\) catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFN\(_\gamma\) antibody) was used to bind secreted IFN\(_\gamma\) and an IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) to bind secreted IL-4. The cell bound IFN\(_\gamma\) and IL-4 was the detected by anti-IFN\(_\gamma\)-FITC and IL-4-PE. Both kits were used in combination on the same sample to detect IFN\(_\gamma\), IL-4 and IFN\(_\gamma\) & IL-4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFN\(_\gamma^+\) and IL-4+ secreting lymphocytes after stimulation with SEB and Ro60. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

Detection of IFN\(_\gamma\) and IL-4 secretion from cells in response to Ro60

PBMC isolated from three age-matched Ro positive and control subjects were used to detect IL-4 and IFN\(_\gamma\) secreting peripheral lymphocytes following a 6hr in vitro stimulation with 5, 10, 20 \(\mu\)g/ml Ro60. Two of the Ro positive subjects had SLE (R7 and R8) and one had SS (R9). One subject was immunosuppressed (R7) the other two (R8 and R9) were not receiving treatment. One Ro positive subject had inactive SLE (R8) and the disease activity for the other Ro positive SLE subject (R7) was not known. The fold change from the non-stimulated control was calculated from the percentages of IFN\(_\gamma\) and IL-4 secreting cells (Table 5.7) after stimulation with Ro60 and SEB and the results for the Ro positive and the control groups are shown in Figure 5.8.
Table 5.7: IFNγ, IL-4 and IFNγ & IL-4 secreting lymphocytes isolated from three Ro positive subjects (R7, R8 and R9) and age and sex-matched controls (C5, C6 and C7) after antigen stimulation with SEB and Ro60 (5μg/ml, 10μg/ml 20μg/ml) following 6 hours incubation. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits in combination. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ and IL-4+ secreting lymphocytes after stimulation with SEB and Ro60. These were compared to unstimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
Figure 5.8: IFNγ and IL-4 secreting lymphocytes isolated from three Ro positive subjects (R7, R8, and R9) and age and sex-matched controls (C5, C6, and C7) after antigen stimulation with SEB and 5, 10, and 20μg/ml Ro60 following 6 hr incubation. IFNγ+ and IL-4 secreting cells were labelled using the MACs IFNγ and IL-4 secretion assay kits. IFNγ and IL-4 capture reagents (CD45 antibody which binds to all leukocytes conjugated to IFNγ or IL-4 antibody) were used to bind secreted IFNγ or IL-4. Bound IFNγ and IL-4 was detected by anti-IFNγ-FITC and IL-4-PE. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
There was no difference between the groups in the IFN\(\gamma\) and IL-4 responses to SEB. In two of three Ro positive subjects the IFN\(\gamma\) secretion was greater than the non-stimulated control (1.3 and 4.6 fold) and also for the control group two subjects (1.8 and 50 fold) were greater than the non-stimulated control. The Ro positive subject with SS (R9) had the highest level of IFN\(\gamma\) secreting cells (both cells secreting IFN\(\gamma\) and also those secreting both IFN\(\gamma\) and IL-4) in response to SEB. None of the Ro positive subjects gave an IL-4 increase to SEB above the non-stimulated control; but the response was greater than the control (2.6 and 6.2 fold) in two of the control subjects. In cells secreting both IFN\(\gamma\) and IL-4, all the control subjects (2.1, 2.9 and 3.4 fold) and one Ro positive subject (3.4 fold) responded to stimulation with SEB.

One Ro positive subject showed IFN\(\gamma\) secretion to 10\(\mu\)g/ml Ro60 greater than the non-stimulated control (1.7 fold) and this was the Ro positive subject with the highest increase to SEB. Again this was the Ro subject with SS and not SLE. The control subject with the 50 fold increase to SEB gave increases to Ro60 at 5, 10 and 20\(\mu\)g/ml Ro60 of 3.3, 3.5 and 4.1 fold respectively.

IL-4 increased compared to the non-stimulated control after stimulation with Ro60 at 10 and 20\(\mu\)g/ml (2.3 and 2.5 fold respectively) in one Ro positive subject (in these cases the corresponding IL-4 level after stimulation with SEB was not greater than the non-stimulated control). This was the SLE subject being treated with immunosuppression. The other two Ro60 subjects did not give an IL-4 level greater than the non-stimulated control. In the control group the highest increases to IL-4 was a 3.2 fold to 5\(\mu\)g/ml Ro60, this subject gave the highest IL-4 response in this group to SEB of 6.2 fold. The highest stimulation with Ro60 compared to the non-stimulated control of cells secreting both IFN\(\gamma\) and IL-4 was 1.9 fold. This was the Ro positive subject with SLE not receiving any immunosuppressive treatment.

The results of IFN\(\gamma\) secretion to SEB using the IFN\(\gamma\) and IL-4 kits in combination were not as high as those observed when measuring IFN\(\gamma\) independently. A reason for this may have been the lower labelling intensity of the FITC conjugated detection antibody used in the combination kit compared to the phycoerythrin (PE) conjugated detection antibody used for the detection of IFN\(\gamma\) only. The manufacturer only recommended the FITC kit for combination measurements when the PE kit could not be used. Also levels of detection of IL-4 detection in response to SEB may be more sensitive when measuring IL-4 alone as was demonstrated in an earlier test.
Detection of IFNγ and IL-4 secretion from cells using separate assays

PBMC were isolated from a Ro positive subject (R5) and control subjects (C8 and C3) and the cells were stimulated for 6 hr and 16 hr in vitro with SEB or 10μg/ml Ro60. The control subjects were included in this experiment to account for any difference in response between the two groups. The percentages (and number) of IFNγ, CD4+ and CD4- secreting cells were measured (Table 5.8) and data expressed as fold change (of % positive cells) compared to the control cells (Figure 5.9). A separate determination was made of the percentage (and number) IL-4 secreting cells (Table 5.9) and data also expressed as fold change (of % positive cells) compared to the control cells (Figure 5.10). In previous experiments the percentage of cytokine secreting cells after stimulation with Ro60 was less than five percent and, so as recommended in the assay instructions, the volume of medium used in the secretion assay was reduced to 1ml.

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Table 5.8 IFNγ+ CD4- and IFNγ+ CD4- secreting lymphocytes isolated from a Ro positive subject (R5) and age and sex-matched controls (C8 and C3) after antigen stimulation with SEB and Ro60 (10μg/ml) following 6 and 16 hours incubation. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ and IL-4+ secreting lymphocytes after stimulation with SEB. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
The IFNγ+ CD4- level after 16 hours stimulation was greater than the non-stimulated control after 6 hours in the subject (R5) and age and sex-matched controls (C8 and C3) although the difference was greater after 16 hours incubation in the subject compared to 10 and 5 fold (r e s p e c t i v e l y ) in the controls. The IFNγ+ CD4- level after 16 hours stimulation was greater than the non-stimulated control after 6 hours in the subject compared to 10 and 5 fold (respectively) in the controls. The IFNγ+ CD4- level after 16 hours stimulation was greater than the non-stimulated control after 6 hours in the subject compared to 10 and 5 fold (respectively) in the controls.

**Figure 5.9** IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes isolated from a Ro positive subject (R5) and age and sex-matched controls (C8 and C3) after antigen stimulation with SEB and Ro60 (10μg/ml) following 6(i) and 16(ii) hours incubation. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Values are fold (of % positive cells) change from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
The IFNγ+, CD4- level after SEB stimulation was greater than the non-stimulated control after 6 and 16 hr incubation in both the Ro positive subject (10.6 and 59.3 fold respectively) and the control subjects (4.8 and 21.7 fold respectively); although the increase was greater after 16 hr incubation in both cases. The IFNγ+, CD4+ level after SEB stimulation was greater than the non-stimulated control in the control subject after 6 and 16 hr incubation (5.0 and 3.2 fold respectively). The Ro positive subject gave a 1.5 fold increase compared to the non-stimulated control after 6 hr, and an 8 fold increase after 16 hr; this was consistent with the lower response after 6 hr compared to 16hr in the IFNγ+, CD4- secreting cells.

Ro60 stimulation compared to the non-stimulated control for IFNγ+, CD4+ secreting cells after 6 and 16 hr in the Ro positive subject was 1.5 and 0.5 fold respectively and in the control subject 1.13 fold after 6 hr incubation the value for 16 hr incubation was not recorded as it was less than the no dye control suggesting that Ro60 did not stimulate IFNγ secretion from CD4+ cells. The IFNγ+ CD4- levels compared to the non-stimulated control after Ro60 stimulation in the Ro positive subject was 8.7 fold after 6 hr incubation compared to 1.8 fold after 16 hr incubation. In the control subject these values were 2.8 fold after 6 hr incubation and 1.0 fold after 16 hr incubation. The increase in IFNγ secretion to Ro60 in both subjects after a 6 hr incubation was less in the control subject than the Ro positive subject (as may be expected) and suggested under these assay conditions an autoimmune response against Ro60 may be stimulated. To further investigate this experiment was repeated under these assay conditions on two further Ro positive subjects and age and sex-matched controls. In this initial experiment the subject was Ro positive SLE and being treated with immunosuppressants, the blood sample was not taken at a regular clinic appointment but the last clinic appointment was within a month of giving blood. At this time the markers of activity were normal although the patient was described as being photosensitive. Since photosensitivity has been associated with the presence of autoantibody to Ro60 this may be a factor affecting the response to Ro60 in this subject.
Table 5.9 IL-4 secreting lymphocytes isolated from a Ro positive subject (R5) and age and sex-matched controls (C10 and C3) after antigen stimulation with SEB and Ro60 (10μg/ml) following 6 and 16 hours incubation. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ and IL-4+ secreting lymphocytes after stimulation with SEB. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

The results of the IL-4 secreting peripheral lymphocytes from a Ro positive subject and control subjects after in vitro stimulation with SEB and Ro60 for 6 and 16hr are shown in figure 5.10. Firstly in the control subject IL-4 secretion increased after stimulation with SEB compared to the non-stimulated control after 6 and 16 hr incubations (3.0 and 5.2 fold respectively). This level was greater after the 16hr incubation as was seen with the IFNγ level. In the Ro positive subject the values were less than the non-stimulated control. Ro60 stimulation did not cause levels to increase above the non-stimulated control in either subject for 6hr or 16hr incubation times.
Figure 5.10 IL-4 secreting lymphocytes isolated from a Ro positive subject (R5) and age and sex-matched controls (C10 and C3) after antigen stimulation with SEB and Ro60 (10µg/ml) following 6(i) and 16(ii) hours incubation. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
5.5.6 IFNγ and IL-4 secretion from cells after stimulation with Ro60 using optimised parameters

The preliminary experiments described in the previous sections showed that using the IFNγ assay and the IL-4 assay separately with a secretion volume of 1ml gave a greater than two fold secretion of the cytokines after stimulation with SEB compared to the control cells, with the exception of the Ro positive peripheral lymphocytes which did not secrete IL-4 in this case. After 6hr incubation a Ro positive subject gave an 8 fold increase in terms of IFNγ CD4- and a lower 2 fold increase was also detected in the control subject. These experimental conditions were used to determine any IFNγ or IL-4 in three age-matched Ro positive and control subjects and the number and % of positive cells are shown in Tables 5.10 and 5.11. Figures 5.11 and 5.12 show fold changes (of % positive cells) in cytokine secretion after stimulation with SEB and Ro60 compared to the non-stimulated control. The Ro positive subjects were diagnosed as having cutaneous LE (R10), neutropenic SS (R11) and SLE (R12). Only subject R12 was on immunosuppressive drug treatment. Immunological markers of disease activity and clinical examination suggested this subject may have active SLE.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Number of gated cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ,CD4-</td>
</tr>
<tr>
<td></td>
<td>B  U  SEB Ro60</td>
</tr>
<tr>
<td>R10</td>
<td>2 (0.06) 67 (1.55) 35 (0.70) 51 (1.58) 3 (0.08) 39 (0.90) 36 (0.72) 44 (1.37)</td>
</tr>
<tr>
<td>R11</td>
<td>2 (0.05) 71 (2.18) 37 (1.72) 103 (2.44) 5 (0.14) 51 (1.57) 45 (2.09) 24 (0.57)</td>
</tr>
<tr>
<td>R12</td>
<td>3 (0.04) 8 (0.11) 25 (0.4) 36 (0.46) 2 (0.03) 43 (0.62) 36 (0.56) 28 (0.36)</td>
</tr>
<tr>
<td>C9</td>
<td>5 (0.09) 17 (0.46) 27 (0.73) 26 (0.37) 0 (0) 28 (0.75) 46 (1.24) 39 (0.56)</td>
</tr>
<tr>
<td>C8</td>
<td>0 (0.00) 23 (0.46) 78 (1.98) 29 (1.30) 2 (0.18) 41 (0.82) 133 (3.37) 20 (0.90)</td>
</tr>
<tr>
<td>C11</td>
<td>0 (0.00) 6 (0.15) 245 (6.89) 61 (1.32) 3 (0.06) 17 (0.43) 451 (12.68) 27 (0.58)</td>
</tr>
</tbody>
</table>

Table 5.10 IFNγ+ CD4- and IFNγ+ CD4- secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8 and C11) after antigen stimulation with SEB and Ro60 10μg/ml for 6 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ CD4- and IFNγ+ CD4-secreting lymphocytes after stimulation with SEB and Ro60. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
Figure 5.11: IFNγ+ CD4- and IFNγ+ CD4- secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8 and C11) after antigen stimulation with SEB and Ro60 10μg/ml for 6 hrs. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. Bound IFNγ was then detected by anti-IFNγ-PE. IFNγ+ , CD4+ cells were detected with anti-CD4-FITC. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
The IFNγ+, CD4- level after stimulation with SEB was greater than the non-stimulated control in one Ro positive subject R12 (5 fold) and all subjects in the control group C9, C8 and C11 (1.7, 4.3 and 45 fold). The IFNγ+, CD4+ was greater than the non-stimulated control in the Ro positive subject R11 (1.95 fold) and again in all control subjects (1.6, 4.9 and 34 fold). The Ro +ve subject with the highest IFNγ+, CD4- increase after stimulation with SEB also the highest increase after stimulation with Ro60 (6 fold) and the control subjects (C8 and C11) also responded to Ro60 (2.8 and 8.8 fold). The IFNγ+, CD4+ levels in the Ro positive group compared to the non-stimulated control were 0.3, 0.56 and 1.56 fold to Ro60 and in the control group the highest responder again was C11 (1.4fold).

Table 5.11 IL-4 secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C10 and C11) after antigen stimulation with SEB and Ro60 10µg/ml for 6 hours. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ CD4- and IFNγ+ CD4-secreting lymphocytes after stimulation with SEB and Ro60. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
Figure 5.12: IL-4 secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C10 and C11) after antigen stimulation with SEB and Ro60 10µg/ml for 6 hours. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.

Five out of six subjects secreted IL-4 after stimulation with SEB when compared to the non-stimulated control (Figure 5.12). These were 1.9, 3.1 and 16 fold in the Ro positive group; 2.2 and 3 fold in the control group. Three subjects secreted IL-4 after stimulation with Ro60 when compared to the non-stimulated control. These were one Ro positive subject with a 4.9 fold response (corresponds to the 3.1 fold SEB response) and two controls with 1.8 and 3.8 fold responses (response to SEB 3.0 and 2.1 respectively). Subject R12 gave the highest IFNγ and IL-4 response to SEB and also gave an IFNγ to Ro60. This SLE subject may have been in an active phase of the disease (Table 5.1D) and this may have an effect on the response to Ro60. Also this subject was on immunosuppressive treatment and this did not appear to suppress the cytokine response to SEB as it was greater than in the two subjects who were not on any treatment. Subject R11 gave the highest IL-4 response to Ro60 this subject had SS and perhaps the cytokine responses to Ro60 are different in SLE and SS.
5.5.7 IFNγ and IL-4 secretion from cells in response to stimulation with ROS damaged Ro60

The second part of the investigation was to see if Ro60, which had been damaged by reactive oxygen species, would stimulate peripheral lymphocytes from Ro positive subjects. The assay conditions used were the same as those described above, namely individual measurement of IFNγ and IL-4 after 6hr incubation with antigen using a 1ml secretion volume in the secretion assays. PBMC from the same three Ro positive subjects as were used in the previous investigation (section 5.5.7). These were Ro positive subjects diagnosed as having cutaneous LE (R10), neutropenic SS (R11) and SLE (R12). Subject R12 was on immunsuppressive drug treatment and immunological markers of disease activity and clinical examination suggested this subject may have active SLE. The reactive oxygen species damaged antigen to be used in these experiments was that which was prepared in chapter 4 by the treatment with H₂O₂ and Cu. A sterile aliquot of Ro60 was treated with filter sterilised H₂O₂ and Cu in a class II cabinet and the treated protein was analysed for the presence of protein carbonyls on an aggregate of Ro60 to show that it had been modified by oxidation (Figure 5.1).

The effect of the PBS treated with H₂O₂ and Cu control on the cytokine response

PBS was treated with H₂O₂ and Cu and this was used as a control to assess the effects of H₂O₂ and Cu on the cytokine response of peripheral lymphocytes from the three age matched Ro positive (R10, R11 and R12) and control subjects (C8, C9 and C11). From the percentages of IFNγ and IL-4 secreting cells detected after stimulation with PBS containing H₂O₂ and Cu (Table 1.12) the fold change from control (no antigen) was calculated and the results for the Ro positive and the control groups are shown in Figure 5.13. The results showed five out of six subjects had a greater IFNγ+, CD4- level after stimulation with PBS containing H₂O₂ and Cu, than the non-stimulated control; the results in the control group were 2.3, 2 and 11 fold and in the Ro positive group 1.8 and 2.9 fold. Only one subject gave an IFNγ+, CD4+ increase greater than in the non-stimulated control of 2.8 fold, this subject was Ro positive. The IL-4 level after stimulation with PBS containing H₂O₂ and Cu was very high in one Ro positive and one control subject (36 and 41 fold respectively), another Ro positive subject had a 2.3 fold increase compared to the non-stimulated control. As in the previous experiment the Ro positive subject R12 gave a high IFNγ and IL-4 reponse to test stimulation with the control and immunosuppressive treatment of the subject and this did not appear to suppress the cytokine response compared the other Ro positive subjects.
Table 5.12 IFN\(\gamma\)+ CD4- and IFN\(\gamma\)+ CD4- secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8/C10 and C11) after antigen stimulation with PBS with H\(_2\)O\(_2\)/Cu 6 hours. IFN\(\gamma\)+ secreting cells were labelled using the MACs IFN\(\gamma\) secretion assay. An IFN\(\gamma\) catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFN\(\gamma\) antibody) was used to bind secreted IFN\(\gamma\). The cell bound IFN\(\gamma\) was the detected by anti-IFN\(\gamma\)-PE. Cells were also stained with anti-CD4-FITC for the detection of IFN\(\gamma\)+, CD4+ cells. In a separate analysis IL-4 secreting cells was labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFN\(\gamma\)+ CD4- and IFN\(\gamma\)+ CD4- and IL-4 secreting lymphocytes after incubation PBS with H\(_2\)O\(_2\)/Cu. These were compared to un-stimulated control cells (U) and control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
Figure 5.13 IFNγ+ CD4- and IFNγ+ CD4- secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8/C10 and C11) after antigen stimulation with PBS with H2O2/Cu 6 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. In a separate analysis IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. Values are fold change (of % positive cells) from the non-stimulated control derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
The effect of ROS damaged Ro60 on the cytokine response

The effect of stimulation of peripheral lymphocytes from three aged matched Ro positive subjects and control subjects, *in vitro* with ROS Ro60 was investigated. In the last experiment some subjects were shown to respond to PBS containing H$_2$O$_2$ and Cu and so the results from the ROS Ro60 treated cells were compared to those treated with PBS containing H$_2$O$_2$ and Cu as well as to the no antigen control. In addition, the results were also compared to those treated with Ro60 to see if oxidative damage to Ro60 altered the response. From the percentages of IFNγ (CD4- and CD4+) and IL-4 secreting cells detected after stimulation with ROS Ro60 (Tables 5.12 and 5.13) the fold change from control no antigen, PBS containing H$_2$O$_2$ and Cu and Ro60 controls were calculated and the results for the Ro positive and the control groups are shown in Figure 5.14 and 5.15.

For the Ro positive subjects the IFNγ+, CD4+ secretion after stimulation with ROS Ro when compared to the non-stimulated control was 1.3, 1.9 and 2.9 fold; when compared to the ROS control and the Ro control only one, different subject, in each group gave a greater increase than the controls (1.4 and 1.3 fold respectively). One subject gave a 1.6 fold IFNγ+, CD4+ increase compared to the no antigen control and the ROS control. For the control subjects the IFNγ+, CD4-secretion after stimulation with ROS Ro was 1.8, 4.3 and 6.7 fold compared to the non-stimulated control; a 2.1 fold to the ROS control and a 2.4 and 1.5 fold increase compared to the Ro control. IFNγ+, CD4+ levels were 2.7 and 2.2 fold compared to the non-stimulated control; 1.6 and 4.2 fold compared to the ROS control and 1.2, 2.4 and 1.6 compared to the Ro control. Ro positive subject R11 had a 10, 4.3 and 2 fold increase in IL-4 secretion when compared to no antigen, ROS and Ro controls. Also in experiment 5.5.6 this subject also gave the highest IL-4 response to SEB and Ro60. This subject had SS and perhaps the cytokine response SS is different to that in SLE. One control subject also had an increased IL-4 secretion compared to all three controls (51, 1.3 and 28 fold respectively) and another control subject had a 2.2 and 2.0 fold response compared to no antigen and ROS controls. Overall response to ROS damaged Ro60 was not observed in either group but some Ro positive and also control subjects showed IFNγ and/or IL-4 response to this damaged protein.
Table 5.13 IFNγ+ CD4- (A) and IFNγ+ CD4+ (B) secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8 and C11) after antigen stimulation with ROS Ro for 6 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was the detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes after incubation with ROS Ro. These were compared to un-stimulated control cells (U), PBS with H₂O₂/Cu incubated control cells (PBS with H₂O₂/Cu) and Ro incubated control cells (Ro). Un-stimulated control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.
Figure 5.14: IFNγ+ CD4- and IFNγ+ CD4+ secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C8 and C11) after antigen stimulation with ROS Ro for 6 hours. IFNγ+ secreting cells were labelled using the MACs IFNγ secretion assay. An IFNγ catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IFNγ antibody) was used to bind secreted IFNγ. The cell bound IFNγ was detected by anti-IFNγ-PE. Cells were also stained with anti-CD4-FITC for the detection of IFNγ+, CD4+ cells. Values are fold change (of % positive cells) from the non-stimulated control, PBS with H2O2/Cu incubated control and cells Ro incubated control cells derived from cytometric analysis used to detect fluorescent labelled cells acquiring 10,000 events from the PBMC population using a live lymphocyte gate.
Table 5.14 IL-4 secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C10 and C11) after antigen stimulation with ROS Ro for 6 hours. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Flow cytometric analysis was used to detect positive cells by acquiring 10000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IL-4 secreting lymphocytes after incubation with ROS Ro. These were compared to un-stimulated control cells (U), PBS with H₂O₂/Cu incubated control cells (PBS with H₂O₂/Cu) and Ro incubated control cells (Ro). Un-stimulated control cells without the addition of either detection antibodies (B) were used to correct for background fluorescence.

<table>
<thead>
<tr>
<th>subjects</th>
<th>B</th>
<th>U</th>
<th>PBS with H₂O₂/Cu</th>
<th>Ro</th>
<th>ROS Ro</th>
</tr>
</thead>
<tbody>
<tr>
<td>R10</td>
<td>2</td>
<td>9</td>
<td>12 (0.34)</td>
<td>15</td>
<td>11 (0.28)</td>
</tr>
<tr>
<td>R11</td>
<td>1</td>
<td>5</td>
<td>9 (0.41)</td>
<td>30</td>
<td>29 (1.64)</td>
</tr>
<tr>
<td>R12</td>
<td>6</td>
<td>7</td>
<td>23 (0.48)</td>
<td>3</td>
<td>3 (0.07)</td>
</tr>
<tr>
<td>C9</td>
<td>10</td>
<td>22</td>
<td>26 (0.52)</td>
<td>11</td>
<td>39 (0.84)</td>
</tr>
<tr>
<td>C10</td>
<td>4</td>
<td>12</td>
<td>9 (0.7)</td>
<td>119</td>
<td>1 (1.25)</td>
</tr>
<tr>
<td>C11</td>
<td>3</td>
<td>12</td>
<td>9 (0.37)</td>
<td>4</td>
<td>14 (0.35)</td>
</tr>
</tbody>
</table>
Figure 5.15: IL-4 secreting lymphocytes isolated from three Ro positive subjects (R10, R11 and R12) and age and sex-matched controls (C9, C10 and C11) after antigen stimulation with ROS Ro for 6 hours. IL-4 secreting cells were labelled using the MACs IL-4 secretion assay. An IL-4 catch reagent (CD45 antibody which binds to all leukocytes conjugated to an IL-4 antibody) was used to bind secreted IL-4. The cell bound IL-4 was the detected by anti-IL-4-PE. Flow cytometric analysis was used to detect positive cells by acquiring 10,000 events from the PBMC population and a lymphocyte gated was used to detect the number and % IL-4 secreting lymphocytes after incubation with ROS Ro. Values are fold change (of % positive cells) from the non-stimulated control, PBS with H2O2/Cu incubated control and cells Ro incubated control cells.
5.6 THE EFFECT OF Ro60 AND ROS MODIFIED Ro60 ON SLE AND SS PBMC CELL DEATH

5.6.1 Flow cytometric analysis of cell death

Data on the percentage of positive annexin V and PI lymphocytes were obtained from the flow cytometer collecting 10,000 events. A typical 2-colour dot plot (annexin V versus PI) from a healthy control subject whose lymphocytes had not been treated with antigen is shown in Figure 5.16. These results demonstrate detection of three populations; viable (B3), early apoptotic (B4), and necrotic (B1, B2) cells and the percentage of cells in each population was detected.

![Flow Cytometric Analysis of Cell Death](image)

Figure 5.16: Flow cytometric analysis of lymphocyte cell death using PBMC isolated from healthy volunteers using Annexin V-FITC and PI staining (n=1). The 2-colour dot plot shows the fluorescence data for Annexin V FITC (emission 519nm detected in channel 1) and PI (emission 617nm detected in channel 3) acquiring 10,000 events set with a live lymphocyte gate. Quadrant B3 of the dot plot reflects viable cells that do not bind Annexin V-FITC or PI. Quadrant B4 shows early apoptotic cells with exposed PS but intact cell membranes binding Annexin V-FITC but excluding PI. Necrotic or apoptotic cells in terminal stages stain both Annexin V-FITC and PI positive and are shown in quadrant B2. A small number of necrotic cells, which are PI positive, are shown in quadrant B1.
5.6.2 Cell death responses of PBMC from Ro60 positive subjects

A comparison was made of percentage of viable, necrotic and apoptotic lymphocytes in cell samples from Ro positive and healthy controls. These cells were incubated *in vitro* for 6 hr but were not treated with antigen. (Table: 5.3). There was more variability in the data obtained from the Ro60 positive group as indicated by the higher standard deviations. There was no difference between the two groups in terms of viable, necrotic and apoptotic cells.

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Ro positive subjects (n=4)</th>
<th>Control subjects (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
<td>65.3±10.0</td>
<td>62.0±6.4</td>
</tr>
<tr>
<td>Necrotic</td>
<td>8.3±5.6</td>
<td>6.0±3.4</td>
</tr>
<tr>
<td>Early Apoptotic</td>
<td>23.1±10.0</td>
<td>26.4±4.2</td>
</tr>
</tbody>
</table>

Table 5.15: Percentage of viable, necrotic and early apoptotic lymphocytes in PBMC isolated from four Ro60 positive subjects (R9, R13, R10 & R12) and four age and sex- matched control subjects (C7, C12, C9, C11) following *in vitro* culture for 6 hrs. Values are means and standard deviations derived from flow cytometry of lymphocyte cell death following Annexin V-FITC and PI staining of isolated PBMC collecting 10,000 events using a live lymphocyte gate.
Healthy volunteer controls  

![Graph](image)

**Figure 5.17:** Individual data on the percentage of early apoptotic lymphocytes in PBMC isolated from six Ro60 positive subjects (R8, R9, R10, R11, R12, R13) and sex-matched control subjects (C5, C7, C9, C8, C11, C12) following *in vitro* culture for 6 hrs. The individual data is given because reduced early apoptosis in 5 of 6 Ro positive subjects was shown compared to healthy volunteers controls but there is no statistical difference. Early apoptotic lymphocytes were detected using flow cytometry where these cells stained positive with Annexin V-FITC. Annexin V-FITC fluorescence data (emission 519nm detected in channel 1) was acquired counting 10,000 events using a live lymphocyte gate.

Although there was no difference between the two groups, the individual data on the percentages of apoptotic cells in Ro60 positive subjects (n=6) showed that in five out of six cases the percentage of apoptotic cells was reduced compared to the controls (Figure 5.17).

### 5.6.3 Cell death responses in subjects after treatment with the antigens SEB and Ro60

Cell death responses were measured after incubation of PBMC for 6hr with either SEB or Ro60 at final concentrations of 10μg/ml. Cells incubated without antigen were included as a control. Data were collected on the percentage of apoptotic, necrotic and viable cells to determine any changes in cell death induced by antigen. The data shown in Figure 5.18 were expressed as fold change from the no antigen control.
Figure 5.18: Viable, early apoptotic and necrotic lymphocytes from PBMC isolated from six Ro60 positive subjects (R8, R9, R10, R11, R12, R13) and age and sex-matched healthy volunteer control subjects (C5, C7, C9, C8, C11, C12) following in vitro culture for 6 hrs with antigens SEB and Ro60 (at a concentration of 10μg/ml). Values are mean and standard deviation of fold change from a non-stimulated control for Ro positive subjects versus healthy volunteer control subjects. Data were derived from percentage of viable, early apoptotic and necrotic lymphocytes analysed by flow cytometry. PBMC were stained with Annexin V-FITC and PI to label early apoptotic and necrotic cell/late apoptotic cells and 10000 events were collected using a live lymphocyte gate.

There was no statistical difference between the two groups in the levels of apoptotic, necrotic and viable cells after incubation with SEB or Ro60. In both groups the mean value of cell necrosis was increased after incubation with SEB compared to the control, 1.3 fold in the Ro positive group and 1.5 fold in the control group. After incubation with Ro60 mean level of apoptotic cells increased (1.2 fold) compared to the control in the Ro positive group and the mean level of necrotic cells increased after incubation with Ro60 in both groups, 1.2 fold in the control group and 2.1 fold in the Ro positive group. The latter was the highest mean fold change in one of the cell death parameters after antigen incubation and individual data is shown in Figure 5.19. This showed the change in cell necrosis after incubation with Ro60 to be increased in five out of six Ro60 positive subjects and in all the Ro60 positive subjects, the fold change in necrosis was greater than, or equal to, the level of change in the control subjects.
Figure 5.19: Necrotic (including late apoptotic) lymphocytes from PBMC isolated from six Ro60 positive subjects (R8, R9, R10, R11, R12, R13) and age and sex- matched healthy volunteer control subjects (C5, C7, C9, C8, C11, C12) following in vitro culture for 6 hrs with Ro60 (at a concentration of 10μg/ml). Values are fold change from the non-stimulated control for Ro positive subjects versus healthy volunteer control subjects, individual subject data are given to highlight a possible trend for increased necrosis (but no statistical difference) particularly in the Ro positive group following incubation with Ro60. Necrotic (including late apoptotic) lymphocytes were detected using flow cytometry where these cells stained positive with PI. PI fluorescence data (emission 617nm detected in channel 3) was acquired counting 10,000 events using a live lymphocyte gate.

5.6.4 The effect of Ro60 concentration on cell death

Additional data was collected following incubation with 5μg/ml or 20μg/ml Ro60 on three age matched Ro60 positive subjects and controls, in order to investigate the effect of Ro60 concentration on cell death responses (Figure 5.20). In two of three Ro positive subjects apoptosis decreased and necrosis increased at 10μg/ml compared to the other concentrations of Ro60. In the control subjects apoptosis levels were not different to the no antigen control and the changes in necrosis were not dependent on the Ro60 concentration.
Figure 5.20: Viable, early apoptotic and necrotic lymphocytes from PBMC isolated from three Ro60 positive subjects (R8, R9, R13) and age and sex-matched healthy volunteer control subjects (C5, C5, C7, C12,) following in vitro culture for 6 hrs with three different concentrations of Ro60 (5pg/ml, 10pg/ml and 20pg/ml) to investigate the effect the dose of Ro60 on cell death response. Individual subject data for Ro positive subjects versus healthy volunteer control subjects are given as fold change from a non-stimulated control. Data were derived from percentage of viable, early apoptotic and necrotic lymphocytes analysed by flow cytometry. PBMC were stained with Annexin V-FITC and PI to label early apoptotic and necrotic cell/late apoptotic cells and 10000 events were collected using a live lymphocyte gate.
5.6.5 Cell death responses after treatment with ROS damaged Ro60

The final antigen to be investigated in this system was ROS damaged Ro60. Ro60 was shown to be damaged by the metal catalyzed oxidation system of $\text{H}_2\text{O}_2$ and Cu resulting in high molecular weight aggregates and protein carbonyl formation. The ROS damaged protein was stored in aliquots at -80°C for use in these experiments with cell samples from human subjects.

Cell death response to stimulation with PBS treated with $\text{H}_2\text{O}_2$ and Cu

The presence of $\text{H}_2\text{O}_2$ and Cu retained in the ROS treated preparation may have generated cellular oxidative stress resulting in an altered cell death response, therefore to take account of this PBS treated with $\text{H}_2\text{O}_2$ and Cu was prepared and also stored to be used as a sample control. The effect of cell samples from Ro60 positive subjects and normal controls after \textit{in vitro} incubation with PBS treated with $\text{H}_2\text{O}_2$ and Cu compared to those without treatment is shown in Figure 5.21. There was no difference between the two groups in cell death responses to PBS containing $\text{H}_2\text{O}_2$ and Cu. In two of three Ro positive subjects the level apoptotic cells increased when treated with PBS containing $\text{H}_2\text{O}_2$ and Cu compared to the non-treated cells. The levels of necrotic cells when treated with PBS containing $\text{H}_2\text{O}_2$ and Cu compared to the non-treated cells was increased in both groups this increase was greater in the control group.

Cell death responses after treatment with ROS damaged Ro60

ROS damaged Ro60 at a final concentration of 10$\mu$g/ml was incubated with 150$\mu$l of cell suspension in 96-well plastic cell culture plates for 6hr at 37°C in an atmosphere of 5 % (v/v) CO$_2$. Data was collected using three age and sex matched Ro60 positive subjects corresponding controls on the percentage of apoptotic, necrotic and viable cells. In the last experiment levels of apoptosis and necrosis were increased after treatment with PBS containing $\text{H}_2\text{O}_2$ and Cu in some subjects. Therefore the results from the ROS Ro60 treated cells were compared to those treated with PBS containing $\text{H}_2\text{O}_2$ and Cu as well as to the no antigen control. In addition, the results were also compared to those treated with Ro60 to see if oxidative damage to Ro60 altered the response. Figure 5.22 shows the results of this experiment. There was no difference between the two groups in the changes induced in apoptosis and necrosis when treated with ROS damaged Ro60. The mean value of necrotic cells when treated with the ROS damaged Ro60 compared to the non-treated cells was increased in both groups and a greater increase was observed in the control group.
Figure 5.21: Viable (V), early apoptotic (A) and necrotic (N) lymphocytes from PBMC isolated from three Ro60 positive subjects (R8, R9, R13) and age and sex-matched healthy volunteer control subjects (C5, C7, C12,) following in vitro Culture for 6 hrs with PBS containing H_2O_2 and Cu to investigate the effect of H_2O_2 and Cu on cell death responses. H_2O_2 and Cu were used to induce oxidative damage to Ro60 (to be used as an antigen in the following experiment) and so as oxidative stress may affect the cell death response, the effect of H_2O_2 and Cu must be investigated as a control. Individual subject data for Ro positive subjects versus healthy volunteer control subjects are given as fold change from a non-stimulated control. Data were derived from percentage of viable, early apoptotic and necrotic lymphocytes analysed by flow cytometry. PBMC were stained with Annexin V-FITC and PI to label early apoptotic and necrotic cell/late apoptotic cells and 10000 events were collected using a live lymphocyte gate.
Table 5.22: Viable, early apoptotic and necrotic lymphocytes from PBMC isolated from three Ro60 positive subjects (R8, R9, R13) and age and sex-matched healthy volunteer control subjects (C5, C7, C12) following in vitro culture for 6 hrs with ROS damaged Ro. Individual subject data for Ro positive subjects versus healthy volunteer control subjects are given as fold change from a non-stimulated control (1), PBS containing H2O2 and Cu (2) and Ro60 controls (3). Data were derived from percentage of viable, early apoptotic and necrotic lymphocytes analysed by flow cytometry. PBMC were stained with Annexin V-FITC and PI to label early apoptotic and necrotic cell/late apoptotic cells and 10,000 events were collected using a live lymphocyte gate.
A possible trend was observed between Ro positive subjects and control subjects in the level of apoptotic cell death when treated with ROS damaged Ro60 compared to those treated with PBS containing H$_2$O$_2$ and Cu when the data was plotted on a smaller scale (Figure 5.22). In the Ro60 positive group the levels of apoptosis decreased in all subjects whereas in the control group the levels increased for two out of three subjects (Figure 5.22).

Figure 5.23: Early apoptotic from PBMC isolated from three Ro60 positive subjects (R8, R9, R13) and age and sex- matched healthy volunteer control subjects (C5, C7, C12,) following in vitro culture for 6 hrs with ROS damaged Ro. Individual subject data for Ro positive subjects versus healthy volunteer control subjects are given as fold PBS containing H$_2$O$_2$. Data were derived from percentage of viable, early apoptotic and necrotic lymphocytes analysed by flow cytometry. PBMC were stained with Annexin V-FITC and PI to label early apoptotic and necrotic cell/late apoptotic cells and 10000 events were collected using a live lymphocyte gate. This data from the previous experiment with enlarged scale to see the individual points shows a possible trend for reduced apoptosis in the Ro positive subjects compared to healthy volunteer controls.
5.7 DISCUSSION

5.7.1 Cell activation-Introduction

Cytokine secretion after stimulation with Ro60 and ROS damaged Ro60 in SLE and SS PBMC and control PBMC was measured using the MACs cytokine secretion assay. This assay was recommended as an effective method to detect activation of antigen specific T-cells. IFNγ, a major Th1 cytokine, has been suggested to play an important role, and may be involved in the pathology of the SLE (Fan and Wuthrich, 1997; Seery et al., 1997; al-Janadi et al., 1993). Therefore, this cytokine was chosen for primary detection. It was also decided to look specifically at cytokine secretion from CD4 T cells as SLE is a T cell dependent autoimmune disease where CD4+ T cells have an important pathogenic role (depletion of CD4+ cells blocks disease onset in mice, and in humans the effects of HIV infection on CD4+ lymphocytes can ameliorate clinical activity). In addition these cells are thought to be involved in the production of the high affinity anti-Ro antibody (Maran et al., 1993). Since evidence of the dominance of a Th1 response in SLE is inconclusive it was also decided to measure IL-4, a major Th2 cytokine, since Th2 cells are mainly involved in stimulating B cells to produce antibody (Parker, 1993).

Ethics approval was granted for the collection of 12 Ro positive SLE or SS subjects from Dr Hassan's Rheumatology clinic at the Leicester Royal Infirmary by Dr Alison Kinder, Clinical Registrar and age- and sex-matched controls were obtained from healthy laboratory and secretarial personnel working in the University of Leicester, Department of Pathology. Weekly clinics saw a maximum of two Ro positive subjects and they were invited to take part in the study. The time and availability of samples was therefore a limiting factor in this study. There were several factors to be assessed for the detection of optimal cytokine secretion. Firstly cell density, during the cytokine secretion period, had to be adjusted to the expected frequency of cytokine secreting cells. This was altered following trial experiments. Secondly the period of incubation required with protein antigens was suggested to be between 6 and 16 hours (Miltenyibiotec, product information) and the minimum and maximum incubations were investigated. Finally, for the antigen concentration, Miltenyibiotec suggested a concentration of 10 μg/ml and additional concentrations of 5 and 20μg/ml were also investigated. The optimisation was further complicated by the measurement of two cytokines and the potential different optimal parameters for each cytokine and a compromise was reached when measuring them simultaneously.
5.7.2 **Cytokine secretion after simulation with SEB**

For the study, 11 Ro positive subjects and age- and sex- matched controls were recruited. After PBMC isolation the number of cells per ml of blood was compared and no difference in the mean value was shown, but the counts were shown to be more variable in the Ro positive group and this may reflect white blood cell abnormalities sometimes associated with SLE (Dubois and Tuffanelli, 1964; Micheal *et al.*, 1951). As it was not known if cytokine secretion may occur after stimulation with Ro60 or ROS damaged Ro60 a positive control was used to show that the assay was working and also to help set the optimal parameters. Using SEB, cells were stimulated non-specifically at concentration of 10μg/ml as recommended in the product information. The majority of Ro positive subjects and control subjects PBMC showed increased IFNγ (CD4- and CD4+) and/ or IL-4 compared to the controls after stimulation with SEB showing that cytokine secretion could be detected using the assay kits. There was no difference in the levels of the secretion between Ro positive subjects and controls and there was considerable variation in the IFNγ and IL-4 secretion levels between subjects in the same group. The overall detection of both cytokines was not enhanced by incubation time but reduction of secretion volume appeared to increase sensitivity.

The treatment of Ro positive subjects with immunosuppressive drugs it was thought might suppress the *in vitro* levels of cytokine secretion. Cells from those subjects on immunosuppressive treatment were not shown have lower levels of cytokine secretion following stimulation with SEB compared to non treated subjects. Some subjects were also on other types of drug treatment and the effects of such treatment may also have effects on *in vitro* immune cells activation. The Ro positive subjects were SLE, SS or MCTD and disease diagnosis may also affect the level and type of *in vitro* cytokine response following stimulation with SEB. Again there was no evidence to suggest a particular pattern of response relating to the diagnosis of disease. For Ro positive subjects with SLE the disease activity may also be a factor affecting the *in vitro* immune cells activation following stimulation. Again there was no consistent finding that those Ro positive SLE subjects with active disease had increased levels of cytokine secretion following stimulation with SEB compared to Ro positive SLE subjects with non-active disease.

The results of SEB stimulated secretion of cytokines suggested that it did not differ between SLE patients and healthy controls. This agreed with similar studies in the literature where phorbol 12-myristate 13-acetate and ionomycin (PMA/I) was mostly used for non-specific stimulation instead of SEB. PMA/I was not recommended for the use in the secretion assay because the stimulus was too strong (Miltenyibiotec, product information) and because of this, it also resulted in the down-
regulation of CD4 (W. Coward, University of Leicester, personal communication) which was also to be measured. The mechanism of signalling by these two stimuli presumably accounts for the different levels of activation. SEB is a toxin from *S. Aureus* which is a superantigen for T cells and PMA/I consists of the phorbol ester PMA which activates protein kinase C signalling pathway and ionomycin (I) from *S. Conglobatus* which is a Ca\(^{2+}\) ionophore and promotes activation via Ca\(^{2+}\) signalling pathways (Sigma, technical information). The literature has also shown there to be no difference between SLE patients and healthy controls in intracellular IFN\(\gamma\) and IL-4 production of T lymphocytes measured in whole blood by flow cytometry after PMA/I stimulation (and without stimulation) (Nagy et al, 2000). This evidence was supported by data on intracellular IFN\(\gamma\) and IL-4 from PMA/I stimulated T cell clones established from PBMC from SLE subjects and controls, but SLE clones were shown to have a reduced ability to secrete IL-10 suggesting that other cytokine dysfunctions may be involved in the development of SLE (Converso et al, 2000).

Another study looked at IFN\(\gamma\) and IL-4 and IL-10 in PBMC by reverse transcriptase polymerase chain reaction (RT-PCR). Since transcriptional control regulates cytokine production this was considered a relevant indicator of cytokine secretion. The high sensitivity of this assay did not require the stimulation of cells to determine cytokine mRNA expression and more closely reflected the *in vivo* situation. The expression of IFN\(\gamma\) and IL-10 transcripts were significantly increased and IL-4 transcripts significantly decreased compared with the healthy controls (Csiszar et al, 2000). The different approach in this study (ie the cells were not stimulated) means the results of this cannot be directly compared to the previous studies or to the findings in this work. Another study showed strong predominance of Th\(_{1}\) was detected by measurement of intracellular cytokine detection after stimulation of PBMC by flow cytometry in patients with lupus nephritis (Masutani et al, 2001), suggesting altered cytokine profiles associated with particular manifestations the disease. Altered cytokine profiles may also be presented in the local area of organ damage eg T cell clones established from the synovial fluid of one patient with an articular flare-up did not produce any Th\(_{2}\) cell cytokines when compared to those established from PBMC (Converso et al, 2000).

### 5.7.3 Cytokine secretion after stimulation with Ro60

After initial measurements of IFN\(\gamma\) CD4+ and CD4- secreting cells after 16 hr stimulation with Ro60 at 10\(\mu\)g/ml there was no IFN\(\gamma\) CD4+ or IFN\(\gamma\) CD4- increase compared to the non-stimulated control in Ro positive subjects (or control subjects). Therefore for the next Ro positive subject recruited, IL-4 was also measured simultaneously. 5\(\mu\)g/ml and 20\(\mu\)g/ml concentrations of Ro60 were included and also 6 hr incubation was added. A dose dependent increase in IL-4 occurred after
6 hr stimulation with 5μg/ml and 20μg/ml Ro60 (2.3 and 2.5 fold respectively). So these conditions were repeated to complete a set of three age- and sex- matched controls. The second two Ro positive subjects did not secrete IL-4 after stimulation with Ro60 but one control subject also secreted IL-4 (3.2 fold) after stimulation with 5μg/ml Ro60. This subject had the highest increases after stimulation with SEB (6.2 fold) in this group and individual patient variation is the likely explanation for the increased secretion of IL-4. A recent study showed one subject out of twenty with severe disease to have a high level of IL-4 following non-specific stimulation (Nagy et al, 2000). The level of IL-4 was shown to decrease after initiation of intensive corticosteroid and cytostatic therapy (Nagy et al, 2000). This highlights that treatments taken by the Ro positive subjects could be masking the true effects of Ro and SEB in terms of cytokine responses. However of the subjects used in our study we found no evidence that cytokine responses were reduced in those subjects on immunosuppressive treatment compared to those receiving no treatment. Of three Ro positive subjects who’s cells did secrete cytokine in response to Ro60 (R5, R7 and R12) all were on immunosuppressive treatment. Two of these subjects (R5 and R12) had symptoms on clinical examination and/or immunological markers of active disease near the time of taking blood, for the third subject the disease activity was unknown. This suggests that the disease severity may be a factor in whether cells respond to Ro60 in vitro. One of these subjects (R5) was said to be photosensitive at the time of giving blood and this is of particular interest as the presence of autoantibody to Ro60 has been demonstrated to correlate with photosensitivity.

Since no significant increase in IFNγ and IL-4 secretion was observed in the Ro positive subjects after stimulation with Ro60, a final set of experiments was carried out using the IFNγ and IL-4 secretion assays separately and again including the CD4 counterstain in the IFNγ assay. The sensitivity may be improved by using single cytokine detection as the interactions or competition for binding sites by the two antibodies may cause problems when detecting both cytokines simultaneously. Initially 6 and 16hr incubation times were tried on one Ro positive and control subject stimulating with 10μg/ml Ro60 and the secretion volume of 1ml was used in accordance with the lowest expected frequency of cytokine secretion. On this occasion CD4- IFNγ secretion was increased compared to the non-stimulated control (8.7 fold) after 6hr incubation and where only a 2 fold increase was observed in the matching control. Again these conditions were repeated to complete a set of three age- and sex- matched controls. The results demonstrated no difference between the Ro positive subjects or control subjects in IFNγ or IL-4 secretion after stimulation with Ro60.
The results of this investigation suggest that recombinant Ro60 does not stimulate IFNγ or IL-4 secretion in Ro positive subjects. There are no reports in the literature relating to production of cytokines after stimulation with Ro60 in SLE. However a similar study was performed to investigate the effect of short-term cultivation of PBMC from SS subjects with recombinant Ro52 and La followed by measurement of IFNγ by enzyme-linked immunospot (ELISPOT). This study found that no increased IFNγ secretion in the SS or control group (Halse et al, 1996). In our study, subjects were recruited on the basis of a positive serological test for Ro and it was assumed that the sera included antibody to Ro60 as well as those to Ro52. A study in SLE was also performed using the C-terminal peptide of amino acids of the SmD1 protein. This peptide forms part of the spliceosomal complex that play essential roles in RNA processing and is considered to be a major target of anti-Sm reactivity in SLE. T-cells derived from immunised lupus mice were analysed in vitro for cytokine production by ELISPOT. Using this antigen in young mice IFNγ, IL-2, TGFβ and IL-1- were shown to increase compared to those immunised with a control peptide and only IFNγ, and IL-2 were increased in older diseased mice (Riemekasten et al, 2003) This demonstrated the possibility of being able to detect changes in cytokine profiles following activation with antigen in SLE models. However further work in human PBMC is warranted.

5.7.4 Cytokine secretion after stimulation with ROS damaged Ro60
The final part of the study was to investigate if ROS damage to the Ro60 antigen was a possible mechanism where by the autoantigenicity to Ro60 was increased. In the last set if experiments in addition to stimulation worth Ro60, PBMC were also stimulated with ROS damaged Ro60 and PBS H₂O₂ and Cu as an additional control. All control subjects and two out of three Ro positive subjects had an increase in CD4- IFNγ after incubation with PBS containing H₂O₂ and Cu compared to the non-stimulated control suggesting oxidative stress in PBMC causes some type of immune activation. To account for this after stimulation with ROS damaged Ro60 values were normalised to this control. As with the results of the Ro stimulation some subjects showed an increase in IFNγ (in CD4- and CD+ cells) and IL-4 but there was no difference between the Ro positive and control subjects. This may suggest that ROS modification of autoantigen is not the mechanism of autoantigenicity, however the level of and type of oxidative damage may be important factors which have not been addressed in this study and our investigation only represented a model system.

In summary there are many factors which may influence the autoimmune response in SLE. The investigation highlights the number of assay parameters and other variables which may affect the
cytokine response to autoantigen in SLE even before the response to antigen is considered for example, the large variation in immune response between individuals, the effects of treatments which may stimulate or suppress the immune system in patients and controls; the manifestations of the disease and local immune responses in areas of organ damage. A large study population with clinical correlations may help to solve these problems. In response to antigen there are also numerous factors to be considered for example, the type of antigen, the nature of the antigen and how it is produced and the concentration at which it may be immunogenic are all points for discussion even before the consideration of whether modification of its structure may play a role in antigenicity. Since IFNγ mRNA expression is increased in unstimulated PBMC from SLE subjects this has supported an alternative hypothesis rather than a marker of antigen activation in SLE (Csiszár et al, 2000); IFNγ up-regulates IgG production by mononuclear cells (Takahashi et al, 1996) and a recent article has described Ro52 as an IFNγ inducible triplicate motif protein associated with membrane proximal structures and a possible role for Ro52 protein in alterations of plasma membranes during cellular activation or apoptosis was suggested (Rhodes et al, 2002).

Although several studies have been done to identify B cell epitopes of Ro60 (Wharen-Herlenius et al, 1999) concluding that the major antigenic region of Ro60 is in the middle region of the protein sequence, data on the T cell epitopes for Ro60 and other SLE autoantigens is very scarce (Decker et al, 2000; Khalii et al, 2001). These T cell epitopes are likely to be different from B cell epitopes, for example, B cell epitopes are suggested to depend on the tertiary structure of the protein perhaps altering during maturation of the immune response or epitope spreading (Itoh et al, 1992; Hirose et al, 1993; Arbuckle et al, 1999) where T cell epitopes are represented by those peptide fragments presented during antigen processing. Therefore new insights on how the immune response develops may be gained from the identification of T-cell epitopes.

5.7.5 Cell death-introduction
The levels of apoptotic, necrotic and viable cells were investigated in PBMC from SLE or SS Ro positive subjects and normal control subjects before and after stimulation with SEB, Ro60 and ROS damaged Ro60. The DNA binding dye, PI, in tandem with annexin V-FITC was used discriminate apoptotic from necrotic cells (Koopman et al, 1994). Early in the apoptotic process extracellular exposure of phosphodidylerine (PS) occurs (Martin et al, 1995) and this binds AnnexinV in a calcium-dependent manner; this can be used to detect early apoptotic cells. Later in the apoptotic process membrane integrity is lost and the PI stain can enter the cell where it binds to DNA. Necrosis can expose PS but membrane function is lost simultaneously and therefore cells will stain
positive for both AnnexinV and PI or PI only. Therefore early apoptotic cells can be discriminated from necrotic cells as they stain positive for annexin V only (Koopman et al, 1994).

5.7.6 Cell death of non-stimulated PBMC

The mean percentage of apoptotic, necrotic and viable PBMC (those which are negative for both stains) from four Ro positive subjects showed no difference when compared to age and sex-matched control subjects. There was more variation in the apoptotic and necrotic cell levels from the Ro positive subjects as shown by the higher standard deviations in this group and this may be because both inadequate and excess apoptosis has been linked with the pathogenesis of SLE. A deficiency in apoptosis has been observed in MRL-lpr/lpr mice which spontaneously develop a SLE-like disease. This was found to be as a result of a defect in Fas receptor involved in cell signalling resulting in apoptotic cell death (Cohen and Eisenberg, 1991). A deficiency in apoptosis may prevent the complete removal of autoreactive T cells in the thymus and increase in apoptosis in vitro has also been demonstrated in lymphocytes from SLE subjects (Emlen et al, 1993). Also increased levels of soluble Fas (which is pro-apoptotic) has been found in SLE subjects about to develop a disease exacerbation suggesting increased apoptosis may occur in vivo in SLE (van Lopik et al, 1999). An increase in apoptosis may lead to increased levels of apoptotic cell, blebs or autoantigens in the circulation; here oxidative stress or other modifications may play a role in increasing the immunogenicity of autoantigen (Cascicola-Rosen et al, 1994). The clearance of apoptotic material from the circulation is also important. In SLE inherited deficiencies in early factors of the complement system have been observed and these factors play a role in the clearance of apoptotic cells. This condition could lead to defective clearance of apoptotic material from the circulation in SLE (Botto, 1998).

Although there was no difference in the mean values of apoptosis between the two groups, individual values showed that in 5 of 6 cases the percentage of apoptotic cells was reduced compared to the control groups. Reports have suggested that apoptosis may be abnormal in autoimmune disease and in the MRL-lpr/lpr lupus mouse, a structural defect in the fas gene results in decreased apoptosis of peripheral lymphocytes (Cohen and Eisenberg, 1991). However when lymphocytes from these mice are cultured in vitro they showed accelerated apoptosis (VanHouten and Budd, 1992). The change in the cell environment from in vivo to in vitro was used as an explanation for this phenomenon, possibly as a result of cytokine withdrawal. However more recently Emlen et al, 1993 have described accelerated in vitro apoptosis of lymphocytes from patients with SLE, which correlated with disease activity, compared to those with RA or from...
normal controls. Higher apoptosis levels were recorded immediately after the cells were isolated and also following in vitro culture suggesting in vitro culture was not an underlying factor of this effect. There was no correlation between those patients with the highest rate of apoptosis recorded immediately after isolation compared to the 24 and 48 hr time points suggesting that accelerated apoptosis did not occur as a consequence of the isolation procedure. Accelerated apoptosis of SLE PBMC was also reported by Lorenz et al, 1997. In this study cells were cultured for 3 days prior to detection but no correlation with disease activity was found. They also found significantly increased rates of apoptosis in PBMC isolated from patients with other autoimmune diseases and this suggests that in RA (as described above) increased apoptosis was not detected because most of the activated T cells migrate to the inflamed joints.

Accelerated apoptosis was not observed in the current study. One factor may be the time that the apoptosis measurements were made. Emlen et al recorded increases in apoptosis immediately after the cells were isolated and this could be explained by increased apoptosis of circulating cells in SLE or because of decreased clearance of circulating apoptotic cells. This may be a different mechanism to those found undergoing apoptosis after in vitro culture for more than 24hr where cytokine withdrawal has been described as a possible explanation. In this investigation cells were isolated and stored overnight at 4°C for practical reasons before cell death and cell activation were investigated after a 6 hr incubation period. Therefore apoptosis was not measured immediately after isolation and would not reflect the initial metabolic state of the isolated cells. Apoptosis was measured after only a short incubation in culture, therefore the effects of prolonged culture in the cells was also not reflected in these cells.

The method of apoptosis detection was also different to the methods applied in other papers. Emlen et al used acridine orange staining to morphologically detect cells undergoing apoptosis whereas Lorenz et al measured the staining intensity with PI of PBMC nuclei. In brief this was based on the fact that the condensed chromatin of apoptotic cells had a lower staining intensity than those with normal chromatin. These methods differ from the method used here which only detected early apoptotic cells and those in the later stages of apoptosis which would have been stained with PI would not be included. However those detected would definitely be apoptotic and could not be mistaken for cells undergoing necrosis. One possible reason for the lower annexin V staining intensity in our study may have been because SLE subjects have been shown to contain antibodies to phosphatidylserine (PS) in their sera (Setty, 2001); this may have bound to PS on the surface of cells undergoing apoptosis thereby blocking the binding of the annexin V detection antibody and in
hindsight one of the other methods may have been more appropriate for this study. The effect of patient treatments on the levels of apoptosis was investigated by Emlen et al., 1994 and showed administration of corticosteroids and cytotoxic drugs did not alter apoptosis of lymphocytes derived from either SLE or RA.

5.6.7 Cell death after stimulation with SEB and recombinant Ro60

After treatment of SLE (or SS) PBMC and control PBMC with SEB, a non specific T cell activator, no difference was seen between the levels of apoptosis and necrosis between the two groups. There was a slight increase in the mean cell necrosis compared to the non stimulated control, 1.3 fold in the Ro positive group and 1.5 fold in the control group. There was no accelerated early apoptosis of SLE (or SS) PBMC after stimulation with SEB and this result agrees with the literature where Lorenz et al also found the percentage of apoptotic cells after SEB stimulation to be unchanged in SLE subjects and controls. After incubation of SLE (or SS) PBMC and control PBMC with recombinant Ro60 no significant difference in early apoptosis or necrosis was observed perhaps, as a result of the small sample number and the individual subject variation. Looking at the individual data after stimulation with recombinant Ro60 the change in cell necrosis after incubation with 10μg/ml Ro60 was increased in five out of six Ro60 positive subjects and in all the Ro60 positive subjects the fold change in necrosis was greater than, or equal to, the level of change in the control subjects. The individual data on levels of apoptosis after stimulation with 5, 10 and 20 μg/ml recombinant Ro60 shows that in all the control subjects the levels of early apoptosis showed no change compared to the non stimulated control where as in the Ro positive subjects the levels were much more variable ranging from a decrease in apoptosis of 0.5 fold to increases of 2 fold. This suggests that recombinant Ro60 has no effect on early apoptosis in normal controls but in Ro positive subjects it may cause increases or decreases in apoptosis depending on the individual subject.

The increase in necrosis in SLE subjects after incubation with recombinant Ro60 is difficult to explain as cellular necrosis is not a programmed event associated with the pathogenesis of the disease. There is little data in the literature relating to antigen induced cell necrosis in immune cells. Increased necrosis could be linked to a change in cell death homeostasis perhaps by decreased apoptosis, eg two out of three Ro positive subjects incubated with 10μg/ml Ro60 apoptosis showed a decreased apoptosis compared to those incubated with 5 and 20μg/ml Ro60 where antigen concentration is likely to affect the levels of apoptosis just as antigen dose can be manipulated to give a maximum activation of T cells. In the same subjects an increase in necrosis was observed.
after incubation with 10μg/ml. Apoptosis was not decreased compared to the no antigen control after stimulation with 10μg/ml Ro60 and this is not a likely explanation for the increase in necrosis. Within those cells detected as necrotic there may also be cells in the later stages of apoptosis; perhaps these represent the increases in cells detected as necrotic, although this is unlikely as cells were only incubated with antigen for 6 hr and the apoptotic process is slow (6-48 hr) (Elkon, 1997). It would be expected that only early apoptotic events associated with antigen stimulation would be detected. Alternatively, activated cell death may result in necrosis in vitro as the normal programme of apoptotic cells death is altered when cells are incubated with antigen under culture conditions.

5.6.8 Cell death after stimulation with ROS damaged recombinant Ro60
ROS damaged Ro60 was also used to stimulate SLE (or SS) PBMC and PBMC from normal control followed by the measurement of the levels of apoptotic and necrotic cells. Firstly, the effect of H₂O₂ and Cu retained in the ROS treated preparation was assessed. There was no difference between the two groups in cell death responses to PBS containing H₂O₂ and Cu. The level of necrotic cells when treated with PBS containing H₂O₂ and Cu compared to the non-treated cells was increased in both groups and this was probably a result of oxidative stress related damage of the PBMC cells resulting in cell injury and death. In addition to attack on membrane lipid from outside the cell H₂O₂ would be able to pass through the cell membrane leading to oxidative changes within the cell. In 2 of 3 Ro positive subjects the level of apoptotic cells increased when treated with PBS containing H₂O₂ and Cu compared to the non-treated cells, possibly indicating that these cells are more susceptible to oxidative damage.

T cell activation has been shown to increase the amount of ROS in T cells but how these extra ROS are generated is unclear (Devadas et al, 2002). As well as generation within T cells via processes of metabolic activity it been postulated ROS may also diffuse into activated T cells following production in neighbouring activated neutrophils (Cauley et al, 2000). Activated T cells have been shown to undergo cell death in culture and this process has been reduced by the addition of antioxidants, suggesting a role for ROS in T cell death (Hildeman et al, 1999). Two mechanisms of death by activated T cells have been identified; these are antigen induced cell death (AICD) mediated predominantly by signalling through Fas (Shi et al, 1989) and activated T cell autonomous death (ACAD) where cells die equally well in the absence of Fas signalling (Hildeman et al, 2002). Several experiments suggest this mechanism is controlled by various members of the Bcl-2 family of proteins. ROS have been implicated in both these processes; antioxidants have been shown to inhibit FasL induction after TCR engagement suggesting a role in AICD (Hildman et al, 2002).
mRNA isolated from activated T cells treated in culture with antioxidants showed mRNA Bcl-2 levels were markedly increased but no change was observed in Fas, FasL or other proapoptotic molecules, implicating ROS in ACAD(Hildeman et al, 2003).

There was no difference in the levels of apoptotic or necrotic cells between SLE (or SS) PBMC and control PBMC after treatment with ROS damaged Ro60 when compared to the no antigen, PBS containing H$_2$O$_2$ and Cu and ROS Ro controls. However individual values showed that levels of apoptosis were decreased in all Ro positive subjects when compared to the PBS containing H$_2$O$_2$ and Cu control but in the control group levels were increased in two out of three subjects. In each of the age and sex-matched subjects the level of apoptosis was decreased in the Ro positive subject compared to the control subject. This data suggests that there may potentially be some difference in the way apoptosis is regulated in SLE in response to ROS damaged Ro60.
CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK
6.0 DISCUSSION

6.1 Introduction
The focus of this thesis was to investigate if protein oxidation induced by oxidative stress could result in enhanced immunogenicity of protein autoantigen and thus be a possible mechanism for the development of autoimmune disease. This hypothesis was tested using a model autoantigen in the prototype autoimmune disease, SLE. The chronic inflammatory response which persists in SLE means cells of the body are subject to oxidative stress as a result of ROS production from inflammatory cells. ROS have been implicated in the development of SLE where DNA has been demonstrated to be highly immunogenic after modification by ROS (Blount et al, 1989). For protein oxidation studies Ro60, a major autoantigen in SLE, was used. For this protein, oxidative insult may result as a consequence of cellular oxidative stress from inflammatory processes (Curi et al, 1998) and in addition oxidative stress may result from UV in sunlight which can also lead to ROS production in cells (Lawley et al, 2000). This may explain the positive correlation between development of photosensitive skin lesions in SLE and the presence of anti-Ro antibody (Mond et al, 1989).

6.2 Production of recombinant Ro60 and modification by ROS in vitro
To investigate this hypothesis a source of recombinant Ro60 was obtained for in vitro protein oxidation studies. The purification procedure was challenging as the protein was found to be highly insoluble when overexpressed in E.coli. However the aim was achieved albeit the protein concentration was relatively low. This limited the techniques that could be applied to detect oxidative changes and immunochemical procedures were applied because of their high sensitivity and relative ease of application, thus minimizing further loss of protein via complex procedures. To investigate if Ro60 was a likely candidate for ROS induced oxidation, UVB and a common ROS generating system (MCO) were used as oxidising sources to detect global protein oxidation products (aggregation, fragmentation and the formation of carbonyls). The methods employed showed no oxidative modifications in vitro to recombinant Ro60 after exposure to a dose range of UVB relevant to environmental exposure. This was, however, a model system and does not rule out the possibility of UV induced modification to Ro60 in vivo. Since metal catalyzed oxidation did result in oxidative changes to Ro60 the work with UVB was not investigated further at this stage. Using the metal catalyzed oxidation (H₂O₂/Cu) with a dose range of H₂O₂ relevant to pathological levels, oxidation induced both the formation of high molecular weight protein aggregates and the formation of protein carbonyl groups on this aggregated protein. The suggested mechanism for these effects
was a 'caged' reaction involving the formation of a copper-amino acid complex and the reaction of H$_2$O$_2$ with this complex causing site-specific damage to the amino acids at a predicted Cu$^{2+}$ binding site on the protein (Stadtman, 1990). This was based on prevention studies using antioxidants where the chelation of Cu$^{2+}$ by DTPA did prevent both aggregation and carbonyl formation but these effects were not prevented using OH scavengers suggesting a free OH was not involved. The same oxidation system was used to study oxidation of lens protein which has been associated with the formation of cataracts. This study demonstrated the presence of high molecular weight aggregates detected with a carbonyl assay. They studied the mechanism of damage by using an OH scavenger and also reported that the radical species involved in this H$_2$O$_2$ /Cu$^{2+}$ was derived from a 'caged' complex (Kato et al, 2001).

6.3 Modification of cellular SLE autoantigens ROS

Metal catalyzed oxidation of lysate from normal dermal fibroblasts also demonstrated high molecular weight protein aggregates and the formation of protein carbonyl groups but whether Ro60 was one of the aggregated proteins remained undetermined, as the carbonyl assay would detect carbonyls formed on any of the lysate proteins susceptible to oxidation. The results suggested the oxidative changes observed might be a common phenomenon using metal catalyzed oxidation. Whether for Ro60 such changes result in altered antigenicity and disease pathology was the focus of further investigation. Metal catalyzed oxidation of lysate from normal dermal fibroblasts also detected the formation of a fragment after treatment that was immunogenic as it was detected by autoantibody presents in SLE sera, suggesting that protein oxidation can result in changes in immunogenicity. Again the origin of this autoantigen could not be identified using these methods and this is an area of future investigation.

6.4 Disease associated with oxidized protein

6.4.1 Aggregation/fragmentation

There is considerable evidence that protein aggregates are involved in the pathogenesis of disease and they have been found to be commonly associated with neurodegenerative disorders. For example in Alzheimer's disease β amyloid protein aggregates accumulate in dense extracellular plaques, which characterize the disease (Tolnay and Probst, 1999. Prion diseases have been shown to be associated with the accumulation of a form of the prion protein which is conformationally modified resulting in protein insolubility and relative resistant to proteases (Prusiner et al, 1998). Also intracellular inclusion bodies (Lewy bodies) mainly containing the aggregated protein α-synuclein have been observed in Parkinson’s disease (Duvoisin, 1996). There appears in some cases
to be a genetic predisposition associated with the development of these aggregates eg two α-synuclein mutations have been identified in cases of Parkinson’s disease and in vitro these mutated forms of the protein have been shown to undergo accelerated aggregate formation compared to the wild type protein (Narhi et al, 1999).

In relation to high molecular weight non-reducible aggregate produced after oxidative damage to the Ro60, similar pertinent observations have been described for the major beta cell autoantigen glutamic acid decarboxylase in type I diabetes mellitus (Trigwell et al, 2001). In this case serum antibodies from type I diabetes patient reacted predominantly with a high molecular weight, modified band suggesting in this case that protein oxidative modification was directly linked to the enhanced immunogenicity of the protein. The aggregated Ro60 was not found to be reactive with SLE serum autoantibodies but the mechanisms of autoantibody production may be different in type I diabetes mellitus to SLE. The results of the high molecular weight aggregate detected after ROS treatment in NDF lysate by carbonyl formation showed the aggregate was observed to contain more than one protein component, of which Ro60 may be present, suggesting possible interactions between proteins were involved in aggregate formation. Proteins susceptible to aggregation have been suggested to draw these other proteins into aggregates via protein-protein interactions especially if a mutant protein interacts more strongly with other protein than does the wild type protein (Kaytor and Warren, 1999). Other critical factors for development of protein aggregates appear to be protein concentration and time as was shown by in vitro studies with the protein huntingtin which forms cytoplasmic aggregates in vivo in the neurodegenerative disorder Huntington disease (Scherzinger et al, 1997).

Studies with SLE serum antibodies did detect an immunoreactive fragment after treatment of NDF cells lysate with ROS, and perhaps purified Ro60 when treated with ROS also produced protein fragments which were beyond the limits of the detection assay due to the low starting concentration of the recombinant Ro60. Fragment formation has been implicated in the pathogenesis of Scleroderma, and like SLE, this is a systemic autoimmune disease where autoantibodies to several cellular components characteristic of the disease are present. The manifestations of this disease are increased vasoreactivity and widespread tissue fibrosis. The striking reversible ischemia-reperfusion that occurs in patients with this disease suggested that this might underlie the injury observed in this disease perhaps involving the generation of pathological levels of ROS. Studies investigating this hypothesis demonstrated that Scleroderma autoantigens were uniquely fragmented by metal-catalyzed oxidation reactions. Interestingly this study showed lupus autoantigens were to be resistant
to fragmentation by this method suggesting this mechanism was specific to the episodes of ischemia-reperfusion unique to this disease (Casciola-Rosen et al, 1997). However in this study the detection of an immunoreactive fragment after treatment of NDF cells lysate with MCO with SLE serum may indicate this to be a potential area for further investigation.

The suggestion that fragments generated by proteolytic cleavage (for example during apoptotic cell death) may be altered in autoimmune states is another area of active research. The cytotoxic lymphocyte granule-induced death pathway has been implicated in the development of systemic autoimmunity. This was based on evidence showing that the proteolytic enzyme Granzyme B, active in this pathway, was able to cleave the majority of autoantigens across the spectrum of systemic autoimmune diseases. Interestingly non-autoantigens were not cleaved by this enzyme or were cleaved to generate fragments identical to those cleaved in other forms of apoptosis (Casciola-Rosen et al, 1999). Fragment formation perhaps by proteolytic processing has also been suggested as an initiating factor in the formation of protein aggregates as seen in many neurodegenerative diseases, for example dense extracellular plaques which characterize Alzheimer's disease and are a result of accumulated β amyloid protein aggregates, have also been shown to contain high levels of a proteolytic fragment derived from the β amyloid precursor protein (Kaytor and Warren, 1999).

6.4.2 Carbonyl formation

In addition to protein aggregation the formation of carbonyls was also detected on oxidized Ro60. Protein carbonyls are one the most abundant markers of protein oxidation and the detection of protein carbonyls is a good indicator of oxidative stress (Berlett and Stadtman, 1998). The carbonyl content of tissues from a variety of species increases dramatically in the last third of lifespan and the accumulation of oxidatively damaged protein is thought to play a role in the aging process (Oliver et al, 1989). In addition, an increase in protein carbonyl is also linked to certain disease states eg. children with juvenile rheumatoid arthritis have been reported to have elevated levels of plasma protein carbonyls compared to control children implicating a role for protein oxidation in the development of certain disease states (Renke et al, 2000). The first studies on protein oxidative modification showed that the introduction of carbonyl groups into a protein resulted in its loss of function followed by its cellular degradation (Levine, 1984) and thus have lead to the understanding that cells rapidly remove proteins damaged by oxidative modification presumably to protect the cell from accumulation of dysfunctional proteins.
6.5 Protein turnover and modified proteins

Subsequent to modification it has been showed that the introduction of carbonyl groups into a protein resulted in its loss of function followed by its cellular degradation. The cellular proteosome has since been shown to be responsible for the increased degradation of the majority of oxidatively damaged proteins (Giulivi and Davies, 1993). However oxidations leading to the formation of protein cross-links have actually been shown to inhibit the activity of the proteosome (Friguet and Swezda, 1997). Enhanced degradation of oxidized proteins is therefore an important mechanism to remove damaged proteins and prevent formation of large aggregates. Aggregate formation and reduced proteosome activity is of major consequences to the cell leading to accumulation of not only aggregated protein but also other damaged or misfolded proteins. For Ro60 further investigations could be performed to determine if the formation of carbonyl groups was the initial oxidative event followed subsequently by protein aggregation. If Ro60 aggregate formation resulted as a consequence of severe oxidation damage this may explain why aggregated Ro60 was no longer recognized by serum antibody to Ro60.

During normal protein turnover, peptide antigen is presented to the immune cells by class I MHC molecules following proteolytic degradation by the proteosome. This ensures the continued maintenance of immune tolerance to "self" protein and the effective clearance of foreign bodies from within cells eg as a result of virus infection. Therefore the accumulation of oxidatively modified protein within the cells may result in the immune recognition of a posttranslational modification, which might direct an inappropriate immune response. Indeed a large number of proteins carry naturally occurring posttranslational modifications and peptides from these modified proteins have been identified as peptide ligands presented by class I and class II MHC molecules in vivo (Dustin et al, 1996; Haurum et al, 1999; Skipper et al, 1996).

Modification of protein may affect T cell recognition of peptide at all levels of antigen presentation. Firstly, modifications of proteins may modify access of proteolytic enzymes thereby leading to the generation of a new epitope to which T cells may not be tolerant or by inhibiting the generation of peptide antigens leading to the accumulation of potentially toxic protein in the cell. Altered proteolysis by granzyme B (a serine protease released by activated immune cells) of a neuronal glutamate receptor leading to autoantigen generation in Rasmussen’s encephalitis was shown to be due to failure of glycosylation of the receptor (Gahring et al, 2001). Another study looking at stability of protein altered by chemical modification and protein antigenicity showed that increasing
protein stability led to depression of T-cell epitope generation by increasing resistance to proteolysis (So et al, 1997).

Antigen presentation at the level of binding to MHC molecules may also be affected by protein modification leading to reduced or increased binding to the MHC molecule. No studies on the effect of ROS modifications on this aspect of antigen processing have been performed. However other posttranslational modifications have been shown to affect antigen processing, for example, glycosylation at a peptide anchor residue required for effective MHC binding was shown to block the binding of the glycopeptide to the MHC molecule (Ishioka et al, 1992). Also a phosphorylated version of a virus derived peptide was found to bind with higher affinity to its MHC molecule than its nonphosphorylated counterpart (Andersen et al, 1999). Finally at the level of T cell recognition, protein modifications may lead to loss of recognition, cross-reactivity or specific recognition of the protein modification. For example, in collagen induced arthritis, immunization of mice with rat type II collagen induces a cross-reactive autoimmune response to type II collagen in cartilage (Michaelsson, et al, 1994). The chemical removal of carbohydrate on the synthetic peptide which induces the response demonstrated that the non-modified peptide blocked T cell recognition of the native peptide. These examples demonstrate that inappropriate protein modifications which may be induced by ROS may have potential consequences for antigen processing at multiple levels and potentially lead to the activation of autoreactive T cells.

6.6 Immunogenicity of ROS damaged Ro60

Immunogenicity of the aggregated Ro60 containing a significantly increased number of carbonyl groups, in terms of T cell activation, was investigated. This was achieved by investigating its ability to induce cytokine secretion (IFN\(\gamma\) and IL-4) as a measure of T cell activation from PBMC obtained from of SLE (and SS) subjects selected for a positive serological test for anti-Ro antibody compared to healthy volunteers. Cell death (apoptosis and necrosis) of PBMC treated with ROS damaged Ro60 was also investigated, as apoptosis is an important factor in regulating activated lymphocyte function and as dysfunctions in the apoptotic process have been linked to the pathogenesis of SLE. Overall, no significant activation of SLE (or SS) PBMC or changes in cell death parameters of lymphocytes after treatment with ROS damaged Ro60 (or the untreated protein) compared to PBMC from normal controls were observed under the experimental conditions applied. This does not mean that ROS damage to protein autoantigen does not play a role in autoimmune disease pathology.
There are a whole spectrum of oxidative changes which may be induced in Ro60 and other SLE autoantigens by several oxidising species. The suggestion that ROS modified autoantigen may stimulate \textit{in vitro} activation of T cells appear not to have investigated and the system used represents a model system which could be modified to look at other oxidative changes. Parts of this study relating to T cell activity agreed with similar studies in the literature, for example \textit{in vitro} IFN\gamma and IL-4 secretion cytokine secretion after non-specific stimulation increased in some subjects compared to the non-stimulated control but there was no difference in levels of secretion between PBMC from SLE (or SS) subjects and normal controls. Similar observations were reported by Nagy \textit{et al}, 2000 and Converso \textit{et al}, 2000. Also to non-modified Ro60 some reactivity to Ro60 in individual SLE and control subjects was shown but again no difference in response was found between the two groups. Here similar observations were found in the literature using recombinant Ro52 and La antigens, indicating an individual susceptibility to antigen stimulation (Halse \textit{et al}, 1996). Interestingly a study by Csiszár \textit{et al}, 2000 did show measurement of IFN\gamma levels in PBMC from SLE subjects to be increased compared to control subjects without any stimulation. This may represent T cells already activated \textit{in vivo} or T cells responding \textit{in vitro} to antigen presented by APC in the sample. In general the lack of response to autoantigen may be because the primed T cells could be more prevalent in the local area of organ damage and extraction of infiltrating cells from this area may represent more appropriate population of cells. Also as T cells react to proteolytic peptides of autoantigen generated during antigen processing and not the whole protein, stimulation with shorter peptides of the autoantigen may be a more effective method for investigating T cell reactivity.

The data on levels of apoptosis and necrosis of PMBC from SLE (or SS) subjects and healthy controls after incubation \textit{in vitro} without antigen, with Ro60 or with ROS damaged Ro60 showed some trends in general relating to a reduced apoptosis in SLE subjects. The levels of apoptosis were shown to be reduced in 6/7 Ro positive subjects compared to matched controls and in 3/3 Ro positive subjects compared to matched control after incubation with ROS damaged Ro60. Apoptosis in SLE is an active area of research with several hypotheses which may have implications for the development of the disease. Defects in the apoptosis signaling pathway have been found in lupus mice and complement deficiency in SLE suggests reduced ability to remove apoptotic cells by phagocytosis, but lymphocytes from patients with SLE have been shown have accelerated apoptosis \textit{in vitro} (Emlen \textit{et al},1993) ; one or all these factors may be involved \textit{in vivo} in SLE. A second trend showed after incubation of PBMC with Ro60 5/6 Ro positive subjects the level of cell necrosis was greater than that of the matched control. There are reports in the literature on levels of necrosis in
SLE the data suggests lymphocytes from SLE subjects may be more susceptible to cellular damage. Overall no statistical difference in the levels of apoptosis and necrosis of PMBC from SLE (or SS) subjects and healthy controls after incubation in vitro without antigen, with Ro60 or with ROS damaged Ro60 were observed perhaps due to individual subject variation and the small sample size of the study.

There is little evidence for the direct effect of protein aggregates on the enhanced immunogenicity of protein. However one study has shown that protein aggregates appear to be involved in the antigenicity of IFNα, where anti-IFNα antibody develops in treatment of subjects with IFNα for various malignant or viral diseases. IFNα protein aggregates were found to be significantly more immunogenic than IFNα monomers (Davies et al, 1997). In addition to aggregate formation, the formation of protein carbonyl was also observed after exposure of Ro60 to ROS. Although this is a very abundant marker of oxidative stress, reports have implicated carbonyl adducts in the altered antigenicity. T cell tolerance to self proteins in mice has been shown to be broken by immunisation with homologous protein, modified using reactive aldehydes which are known to be generated during lipid peroxidation in cells (Wuttge et al, 1999). It appears that the degradation of protein within cells and its effective removal by the immune system are vital processes for the prevention of disease. ROS represent one mechanism whereby damage to protein may occur and it is essential that this damaged protein in effectively cleared from the body as it has the potential to initiate development of disease. This may involve interactions with other cellular macromolecules eg lipid and DNA and have consequences for both the internal integrity of the cells and also for the immune system whereby damaged molecules may no longer be recognized as self.
6.7 FUTURE WORK

6.7.1 Purification of SLE autoantigens
A method was developed to purify soluble recombinant Ro60 overexpressed in E.coli. Since a renaturation procedure was applied it may be assumed that the purified protein was refolded but the conformational state of the protein was not investigated. Since ROS may cause damage associated with protein conformational changes the protein conformation of the undamaged protein may be considered an important issue. The use of an alternative expression system could be investigated to see if Ro60 is still produced in its native form. The human Ro60 gene has been transfected and expressed in HEp-2 cells and this may represent an alternative way of obtaining purified recombinant Ro60. This may also have the advantage of obtaining recombinant Ro60 at high concentration so that this would not be a limiting factor in the subsequent assays to be used. It is also possible that Ro60 is posttranslationally modified in vivo. These modifications (which would not be present when overexpressing in a prokaryotic system) should be achieved by overexpression in a eukaryotic system thus making the protein which more closely resembles that produced in vivo. Failing the production of a high concentration of native Ro60 by this alternative method, other protein autoantigens could be used instead of Ro60. For example, La, another major autoantigen in SLE, is available for overexpression in E.coli but unlike Ro60 this protein accumulates in the soluble fraction after overexpression.

6.7.2 ROS damage to SLE autoantigens
The area of protein oxidation is an expansive field with numerous types of oxidising species and adducts of oxidation, and much research is currently being undertaken to develop reliable methods for the detection of these oxidative products. With unlimited Ro60 starting material of high integrity several oxidising species could be used to generate oxidative changes to Ro60 using a variety of techniques in order to build a picture of the spectrum of oxidative changes to Ro60. In addition to metal catalyzed oxidation and UVB, hypochlorous acid which is produced by activated neutrophils, and nitric oxide, which plays a significant role in the inflammatory process and has been implicated in several autoimmune disorders, may be interesting oxidising sources to investigate. The detection of protein damage by UV could be further investigated by using a monochromatic source of UV to investigate if UV induced oxidation of protein is associated with specific wavelengths.

Among the analytical techniques which could be applied are the measurements of protein hydroperoxides. These can be induced by oxidation on several amino acids and after reduction to
stable hydroxides can be detected by HPLC. Oxidation of aromatic residues, a target of UV induced oxidation, could be measured by the detection of N-formyl kynurenine and L-DOPA also by using HPLC. Some references in the literature relate to the use of a di-tyrosine antibody to detect di-tyrosine cross-links, although a commercial antibody does not yet appear available. The effects of nitric oxide leads to the formation of 3-nitrotyrosine via peroxynitrite and this can be detected also by HPLC or immunochemically using an anti-nitrotyrosine antibody. The product chlorotyrosine resulting from protein oxidation by hypochlorous acid can be detected by HPLC or GC-MS. The extent of oxidation as measured by the formation of protein carbonyls could be further investigated by using an ELISA method instead of Western blotting, and by applying carbonyl standards the number of carbonyl groups could be quantified. However there may be concerns as to whether oxidised protein would bind to the ELISA plate with the same efficiency as the control protein creating possibility of technical errors.

6.7.3 Immune cell response to autoantigen
The characteristic features of SLE are clinically diverse and the immune abnormalities associated with the disease are highly complex. It is therefore difficult to identify factors relating to the response to a particular autoantigen in the disease process as a whole. The ideal situation would be a detectable positive response to an autoantigen in vitro where the antigen could be manipulated and the corresponding changes in immunoreactivity investigated. However a single factor may be involved in modifying the antigen resulting in a switch between inactive and active response and considerable investigation may be required to identify this. PBMC from SLE subjects and controls have been used for cDNA arrays to characterize potentially relevant genes involved in the pathogenesis of SLE. Perhaps this technique could be extended to look at genes involved in cell activation and cell death in PBMC from SLE subjects and controls after treatment with and without potential autoantigens. This would help screen for potential genes expressed in response to treatment with antigen, be they involved in cell activation or cell death.

6.2.4 Antigen specific CD4 T cells
Autoantibody generation to Ro60 and also other autoantibodies in SLE requires T cell help, the epitopes recognized by these T helper cells are not known and this could be a future aim following this study. The detection of live antigen specific T cells to Ro60 using the MAC cytokine secretion assay would have lead to the potential to isolate such cells and to analysis of T cell receptor repertoire and the mapping of T cell receptor epitopes. This was not accomplished using
recombinant Ro60 or ROS damaged Ro60. The method could still be applied to different antigen preparations of recombinant Ro60 with alternative oxidative modifications or other candidate antigens. An alternative approach to determine T cell epitopes may be to isolate peptides by acid extraction from affinity purified MHC class II molecules and determine sequence information by mass spectrometry. This technique has been used to identify potential T cell epitopes for autoantibody production in murine SLE and this study could be applied to human SLE and control PBMC perhaps before and after antigen treatment which might increase the chance of detecting epitopes of a particular autoantigen.

The failure to detect T cell activation after Ro60 stimulation may not mean that these cells were not activated. An alternative approach may be to try to culture Ro60 specific CD4 T cell lines. Starting with purified CD4 T cells and irradiated autologous EBV-transformed B-cell lines as a source of antigen presentation cells, the antigen specific cell could then be selected for proliferation by stimulation with Ro60 or modified Ro60 and IL-2. This may help by elimination of the responses of other cells in the PBMC preparation which are not thought to be involved in this response. The generation of Ro60 antigen specific CD4 T cell lines would be valuable for additional studies for example looking at the process of antigen processing to see if it is altered by the presence of modified Ro60.
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