Autoantigen Specific T cell Responses in relation to Systemic Lupus Erythematosus

by

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Synopsis

Systemic lupus erythematosus (SLE) is an autoimmune diseases characterised by serum autoantibodies. Autoantibodies are IgG class switched and often HLA associated suggesting Th cells are involved in responses directed to proteins to which autoantibodies develop. The aim of this study was to investigate T cell responses to the autoantigens La and β_2 glycoprotein 1 (β_2GP1) in patients with SLE.

Patients showed a reduced proliferative response to recall and naive antigens compared to healthy individuals. Anti - CD28 antibody, nor resting the cells prior to stimulation, restored the responses to levels seen in healthy individuals. Tetanus toxin vaccination before and after clinical diagnosis did not affect the response to tetanus toxoid.

Proliferative responses to β_2GP1 were identified in patients irrespective of serum antibodies, HLA haplotype or clinical diagnosis. PBMC's from healthy individuals did not show proliferative responses to β_2GP1 suggesting that responses are disease associated.

Proliferative responses to synthetic La peptide pools were identified. Quantitative differences in the response between healthy individuals and patients supports the hypothesis of a low level primed response in patients although further work is required. Peptide 49 - 63 was the immunodominant stimulatory peptide although responses could not be differentiated between healthy individuals and patients.

To Martin and my family

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List of Abbreviations

Ab	antibody
ACL	anticardiolipin antibody
ARC	Arthritis Research Campaign
APA	anti - phospholipid antibodies
APC	antigen presenting cell
APS	anti - phospholipid syndrome
β_2GP1	β_2 glycoprotein 1
BCIP	5-bomo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CD	cluster of differentiation
CMI	cell mediated immunity
CMV	cytomegalovirus
CPM	counts per minute
DC	dendritic cell
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
FCM	fibroblast condition medium
FCS	foetal calf serum
FDC	follicular dendritic cell
GAD	glutamic acid decarboxylase
NHS	normal human serum
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
HRP	horse raddish peroxidase
Hsp65	heat shock protein 65
HTLV	human T cell leukaemic virus
IDDM	insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
LAC	lupus anticoagulant
LDA	limiting dilution assay
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MHC	major histocompatibility complex
MIIC	MHC compartment
MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
MW	molecular weight
NBT	nitroblue tetrazolium
NOD	non - obese diabetic mice
NP40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PHA	phytohaemagglutinin
PLP	phospholipid protein
PMA	phorbal myristate acetate
PMSF	phenylmethylsulphonyl fluoride

PPD	partially purified protein derivative of Tuberculin
PS	phosphatidylserine
PWM	pokeweed mitogen
RNA	ribonucleic acid
RNP	ribonucleoprotein
SDS	sodium dodecyl sulphate
SI	stimulation index
SLE	systemic lupus erythematosus
SS	sjogren's syndrome
ssDNA	single stranded DNA
Tc	cytotoxic T cell
TCR	T cell receptor
Th	T helper cell
TEMED	N, N, N', N'-Tetramethylethylene Diamine
TNF	tumour necrosis factor
tRNA	transfer RNA
Ts	T suppressor cell
TT	tetanus toxoid
VZV	vesicular stomatitis virus

Contents

Chapter 1

Introduction

This introduction considers the fundamental principles upon which the research is based. The first section discusses the role of T cells and their interactions within the immune system. The second section describes the potential mechanisms of autoimmunity, while the third section describes the clinical features of the autoimmune disease, systemic lupus erythematosus. The final section describes the immunology of systemic lupus erythematosus including the appropriate animal model systems.

1.1 The immune system

The immune system comprises of specialised cells which have, according to the classic dogma, evolved to protect the organism from foreign antigens (non - self) which invade it. Alternatively, it has been postulated that these specialised cells have a role in discriminating between 'danger' and 'non danger' signals (Matzinger, 1994). These specialised cells include 1) B cells, which produce antibodies, 2) T cells which are involved in the activation of other cell types and 3) antigen presenting cells (APC's). APC's, which include macrophages, B cells and dendritic cells, pick up antigen from the environment, process it in specialised intracellular compartments and present the peptides on their surface in the context of a molecule called the major histocompatibility complex (MHC). T cells then recognise the peptide and the MHC molecule through a surface T cell receptor (TCR) and initiate various effector functions including cytokine production (Figure 1.1 and 1.2). Normally the processes which enable the immune system to distinguish between self and non self peptides bound to the MHC are tightly controlled. However, when the control mechanisms fail antibodies to self proteins may be generated which later may manifest as an autoimmune disease. The mechanisms involved are poorly understood but the high titer, affinity and class switching of the autoantibodies suggests that it is an antigen dependent process requiring the presentation of an autoantigen in a MHC binding cleft to a T cell.

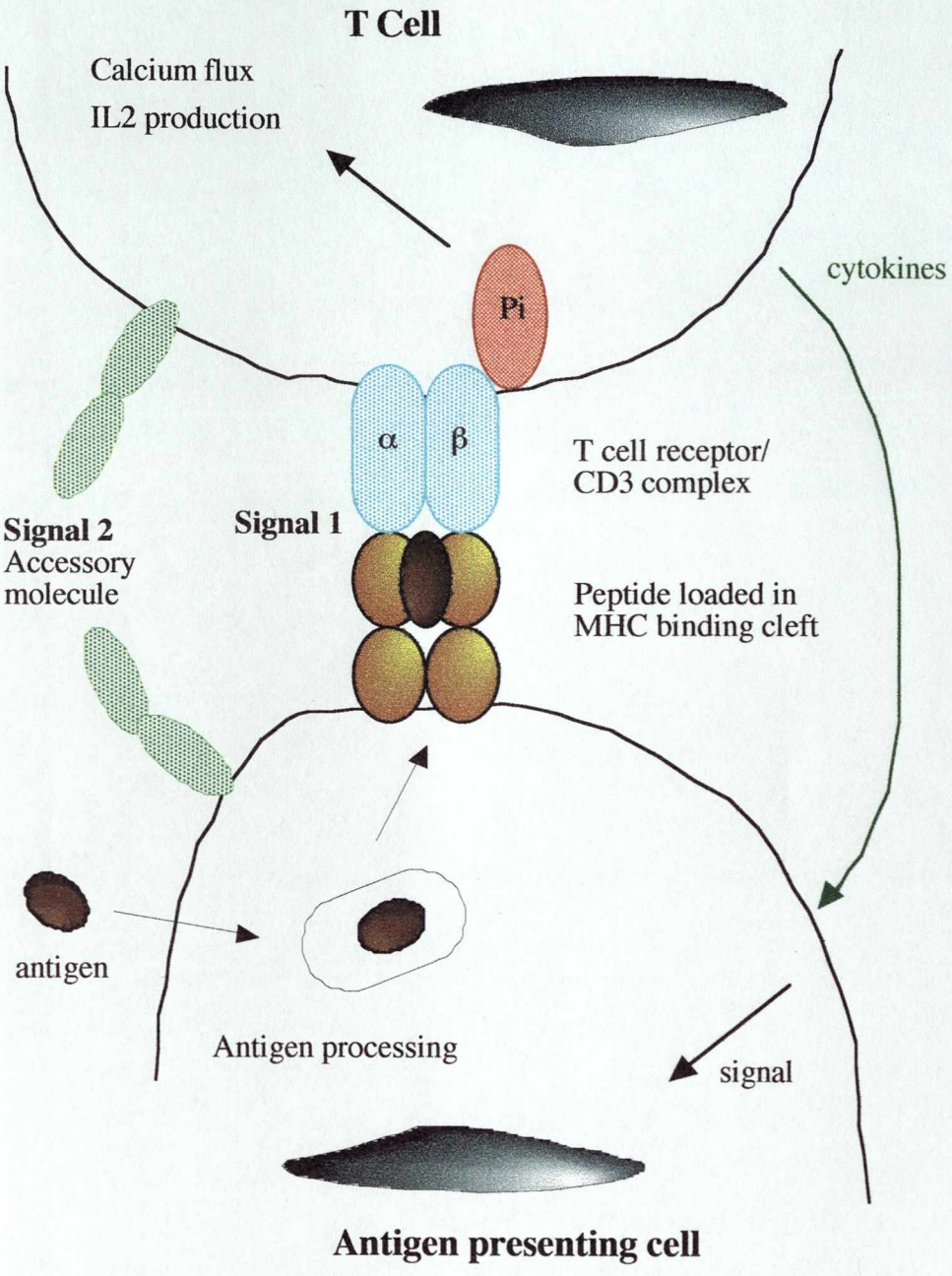


Figure 1.1

Diagrammatic representation of the interaction between a T cell and an antigen presenting cell.

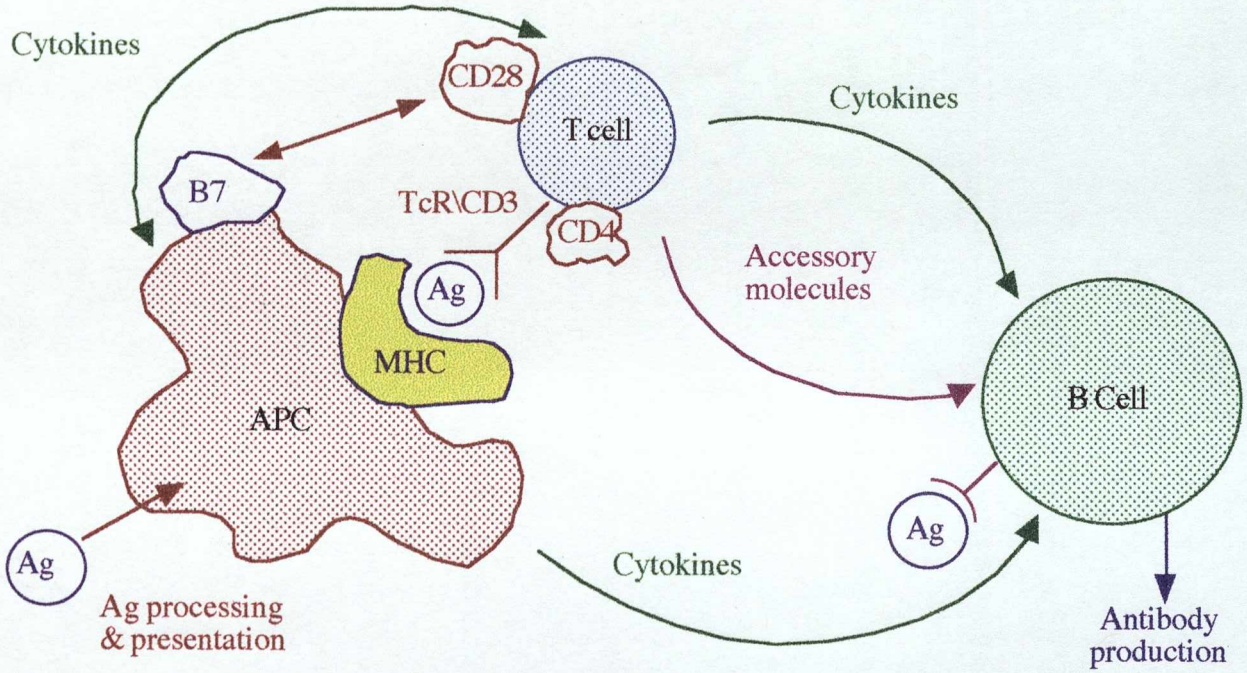


Figure 1.2

T cell Dependent Antibody Production

1.1.1 T cells

T cells are central to the immune response since they recognise degraded protein presented by the APC and initiate effector functions. These effector functions include cell activation, cell proliferation and differentiation and cytokine production (Kumar *et al.*, 1989; Sprent *et al.*, 1990). T cells which recognise MHC class II molecules and co-express the CD4 surface marker are referred to as T helper cells (Th). T cells which recognise MHC class I molecules and co - express the CD8 surface marker are referred to as cytotoxic T cells (Tc). CD4 T cells consist of 65% of all total peripheral T cells with CD8 T cells making up the remainder. T cells which have not encountered antigen before are referred to as naive cells while those that have previously received antigen stimulation are referred to as primed or memory cells. Following the initial encounter with antigen it takes approximately 7 - 8 days to develop a T cell response. However, the T cell response to a challenge with a previously encountered antigen is more specific and vigorous due to the presence of memory T cells. Responses then develop within 5 days. Naive T cells have a half life of 3 - 4 months while activated T cells only live for a few days due to the upregulation of surface Fas and TNF receptor molecules (Croft *et al.*, 1994). Expression of Fas makes the cells prone to apoptosis.

1.1.2 The T cell receptor

T cells recognise peptide bound in the MHC cleft through a surface receptor, referred to as the TCR. The TCR is a disulphide linked heterodimeric transmembrane glycoprotein consisting of either an α and β chain or a γ and δ chain (Kumar *et al.*, 1989). T cells expressing the α and β chain (TCR$\alpha\beta$) make up approximately 95% of the T cell population while T cells expressing the γ and δ chain (TCR$\gamma\delta$) make up the remainder. Each chain of the TCR consists of a variable region, which generates the antigen binding cleft, and a constant region which is involved in effector functions. The TCR variable region is assembled during T cell development in the thymus from variable (V), diversity (D) and joining (J) gene segments in the case of the β and δ chain and from variable and joining gene segments for the α and γ chain (Kumar *et al.*, 1989). Multiple germline segments

have been identified and a random selection of these gene segments generates diversity in the TCR (Davis and Bjorkman, 1988). Recombination of the gene segments occurs until a successful TCR is expressed on the cell surface. This recombination is considered to be the most important event for T cell commitment since the diversity of the variable domain is essential for the recognition of antigens. The TCR is unable to distinguish between specific amino acid residues but recognises amino acid characteristics, including charge and size. Hence, one TCR may potentially recognise multiple peptides with the same characteristics but different amino acid residues (Ignatowicz *et al.*, 1997). However, it must be appreciated that this random generation of the TCR may also potentially produce a TCR which recognises self antigen.

1.1.3 T cell accessory molecules

Contact between a T cell and an APC is a dynamic process which requires the involvement of adhesion, coreceptor and costimulatory molecules for activation. To achieve maximum T cell activation and hence IL2 production and proliferation, two signals are required. The first signal is generated through the interaction of the TCR with peptide bound to the MHC molecule (Weiss, 1993). The second 'costimulatory' signal is delivered through 'accessory molecules' (Geppert *et al.*, 1990). Naive T cells are more dependent on a second signal than primed T cells. The activation status of primed T cells affects the amount of costimulation required (Yi-qun *et al.*, 1996).

The TCR cannot signal into the cell since it does not have the appropriate cytoplasmic tail. Signals through the TCR are generated by CD3 to which the TCR is complexed. CD3 is a 5 chain non polymorphic structure consisting of γ, δ, ε and ζ or ζ-η chains (Weiss, 1993). T cell activation leads to rapid tyrosine phosphorylation of CD3 which initiates a signalling cascade leading to gene transcription and increases in intracellular calcium levels (Samelson and Klausner, 1992). Secondary accessory molecules including CD4 and CD8 stabilise the TCR/CD3 complex interaction on antigen recognition and provide signals into the cell to synergistically trigger signalling events (Weiss and Littman, 1994).

CD4 T cells are divided into subgroups depending on which CD45 isoform is also expressed (Clement, 1992). Naive T cells which have not been exposed to a specific antigen express CD45RA while activated and primed T cells express CD45RO (Akbar *et al.*, 1988). However, increasing evidence supports the hypothesis that immune memory also resides in CD45RA T cells and that CD45RO memory T cells may revert back to the CD45RA expressing cells in the absence of the antigen (Pilling *et al.*, 1996). In this instance the TCR specificity of the 'revertant' cell is unclear. CD45RO T cells are associated with the downregulation of a proto-oncogene bcl-2, a gene which is known to protect cells from entering apoptosis. A gradual loss of bcl-2 is accompanied by a reduced expression of CD45RB, another isotype of CD45 and a marker for cell differentiation (Salmon *et al.*, 1994). Resistance to apoptosis by maintaining bcl-2 is assisted by stromal cell factors including those generated by fibroblasts (Akbar *et al.*, 1993). T cells memory may persist for decades due to the proliferation of T cells derived from the same clone of primed cells (Cerottini and MacDonald 1989). However, memory T cells require constant exposure to MHC bound with peptide for survival (Markiewicz *et al.*, 1998).

Other accessory molecules found on T cells include CD2 (which binds LFA-3 on effector cells) help to bring the T cell and APC closer and to transduce signals into the cell (Shaw *et al.*, 1986). LFA-1 on T cells binding to ICAM-1 on vascular endothelium has a similar function. CD28, which is constitutively expressed on resting CD4 T cells and upregulated by mitogenic stimulation, interacts with CD80 (B7.1) or CD86 (B7.2) on APC (Chambers and Allison, 1997). This interaction leads to the upregulation of the IL2 receptor and stabilises IL2 mRNA to prolong the cellular activation signals (Linsley *et al.*, 1991). Hence, the interaction of CD28 with CD80 or CD86 is important for T cell activation and survival and can prevent anergy (Harding *et al.*, 1992). Furthermore, CD28 deficient mice show a reduced T cell response to antigen (Shahinian *et al.*, 1993). However, Manickasingham *et al.* (1998) report that optimum signalling through the TCR/CD3 complex of naive T cells is sufficient to induce proliferation without the requirement for CD28 interaction.

1.1.4 T cell activation

Within several hours of a TCR interacting with a specific peptide bound in the groove of the MHC molecule the T cell becomes activated. Some activated T cells differentiate into long lived memory cells during which the specificity of the TCR does not change (Ignatowicz *et al.*, 1996). Alternatively, activation leads to an upregulation of surface receptors and production of soluble cytokines. T cells can be subdivided on the basis of cytokine production, first described in mouse T cell lines (Mosmann and Sad, 1996). Cells which secrete IL2, IFNγ and TNFβ are referred to as Th1 type cells. They mediate cell mediated immunity (CMI) and are associated with organ specific autoimmunity. Cells secreting IL4, IL5, IL6 and IL10 are referred to as Th2 type cells and drive antibody production, particularly IgE, in response to allergens and extracellular pathogens. T cells which do not fit into either the Th1 or Th2 classification are termed Th0 type cells and secrete a mixture of cytokines. IL2 promotes B cell growth and T cell activation, particularly Th1 development, while IL4 promotes Th2 development. Hence, cytokines bias subsequent immune responses but they may also non specifically stimulate neighbouring cells in a process referred to as the 'bystander effect'.

1.1.5 Major histocompatibility complex

MHC molecules regulate the immune response through the presentation of peptides to T cells. Particular amino acids of the MHC molecule are important for interacting with the peptides while other regions of the MHC molecule are important for contact with the TCR. Only antigens capable of being presented by self MHC molecules are capable of inducing an immune response in an individual. MHC class I molecules (HLA - A, B and C) are comprised of an α chain, which forms the peptide binding cleft, and a β2 microglobulin molecule which stabilises the structure once peptide has bound. The MHC pocket restricts the size of the peptides which can bind. Peptides are usually 8-15 amino acid in length. Class II molecules (HLA - DR, DP, DQ) comprise two noncovalently associated integral membrane polypeptide chains, an α and β chain. Both chains form the peptide binding groove which can accommodate peptides between 12 and 25 amino acid residues in length

(Kreiger *et al.,* 1991). The open structure of the groove allows protruding flanking amino acids to exert an effect on the T cell response although these extra amino acids do not contribute directly to the T cell specificity.

The MHC genes show extensive polymorphism, characterised by the presence of many alleles. A combination of particular alleles for each class I and class II molecule describes the HLA haplotype. Certain HLA haplotypes are associated with particular populations and are inherited by non random association of alleles at different loci due to close linkage ('linkage disequilibrium'). This may reflect the pathogens found in the local environment. HLA haplotype is determined by serology using specific antibodies or by PCR using specific sequence primers to known polymorphisms. Each methodology of HLA typing has its own nomenclature. A single number following the HLA class (i.e. HLA DR4 or HLA DQ6) describes a collection of alleles with shared serological determinants. These determinants can be identified more specifically with PCR and designated HLA DRβ1*0401 to DRβ1*0407 or DQβ1*0601 to DQβ1*0604 respectively. Since the MHC molecules present antigen to T cells the development of an autoimmune disease may be influenced by the HLA haplotype. For example, ankylosing spondyliltis is associated with HLA B27, rheumatoid arthritis with HLA DR4 and systemic lupus erythematosus with HLA DR3. However, the possession of a autoimmune associated haplotype does not automatically confer to the development of the autoimmune disease (McDevitt, 1998).

Following the construction of the MHC molecule in the endoplasmic reticulum of the APC the binding groove is occupied by an invariant chain peptide (Ii). The tail of the invariant chain signals to move the MHC molecule into the MHC compartment (MIIC). In the MIIC the Ii is degraded all but a small segment which remains bound to the MHC. This segment is referred to as CLIP (class II associated Ii peptide) and is removed by HLA-DM (Pieters, 1997). Endosomes containing protein which has been internalised into the cell and degraded fuse with the MIIC. The CLIP is then removed allowing the peptide to bind the MHC cleft (Kropshofer *et al.*, 1997). The MHC / peptide complex is then conveyed to the cell surface. Most of the peptides bound to MHC molecules are derived from self proteins but not all peptides generated *in vivo* which bind MHC class II will be presented

(Rudensky *et al.*, 1991). It is possible to generate T cell responses to exogenously added peptides *in vitro* which bypass the requirement for antigen processing. This technique has been used to study peptide specific proliferative responses by T cell clones (Lohmann *et al.*, 1996) and freshly isolated peripheral blood cells (Reece *et al.*, 1994). The MHC - peptide - T cell interaction is still being characterised but the T cell repertoire contains no obvious mechanism that limits which peptides are recognised (Panina-Bordignon *et al.*, 1989).

It has been hypothesised that T cells recognise antigen presented by CD1 molecules (Porcelli *et al.*, 1998). CD1 is a MHC-like molecule with a large hydrophobic binding pocket which is electrostatically neutral (Zeng *et al.*, 1987). Recent evidence shows that the pocket can bind and present non - peptide lipids and glycolipids to T cells (Porcelli *et al.*, 1998). However, the associated processing pathway and the interaction with T cells remains to be resolved.

1.1.6 Interaction of APCs with T cells in secondary lymphoid tissue

T cells enter the periphery in a naive state and survive as resting T cells. They recirculate around the body passing through secondary lymphoid organs including tonsils, spleen and Peyer's patches where they sample the environment for antigens which bind to their TCR. Recirculating T cells which are capable of binding antigen presented by APCs become activated and move to areas of the tissue called 'T zones'. Here they interact with specific B cells (MacLennan *et al.*, 1997).

B cells, which are produced in the bone marrow and express surface IgM, recirculate around the body passing through secondary lymphoid tissues. If they do not encounter antigen whilst in the lymphoid tissue they return to the circulation. Such cells die within one month. Some B cells encounter specific antigen but leave the tissue and continue to secrete IgM antibody of low affinity. Those B cells which receive signals from helper T cells move to an area within the tissue designated the T zone or to follicles within the secondary lymphoid tissue.

Follicles consist of a network of follicular dendritic cells (FDC's) which are capable of holding antigen on their surface for years although complement depletion inhibits immune complex formation on the FDC's. B cells take up antigen from the FDC through their surface immunoglobulin, process it and present it to specific T cells in the context of a MHC molecule. This cognate interaction initiates somatic hypermutation of the Ig variable region gene. B cells undergo clonal expansion and form structures called germinal centres consisting of dark and light regions. The dark region represents B cells which are undergoing proliferation and are termed centroblasts. Many of these cells die by apoptosis unless they are rescued by signals through CD40, ICAM-1, MHC and from the environment (Tsubata *et al.*, 1993; Nishioka and Lipsky, 1994) . Centroblasts receiving the correct signals, and which express correctly formed immunoglobulin on their surface, move into the light zone where they differentiate into either long lived memory cells or plasma cells. Plasma cells secrete mainly IgG although the time and location of germinal centre formation has a major impact on the class and subclass of immunoglobulin produced (Toellner *et al.*, 1996). Germinal centres persist for three weeks following immunisation but with successive immunisations their size diminishes.

Follicles within secondary lymphoid tissue are the only site where B cell activation is known to occur. However, B cells may divide without further T cell help once a cognate interaction has occurred and the antibodies generated are of high affinity and class switched (Opera and Perelson, 1996).

1.2 Tolerance

T cells are derived from pluripotent stem cells which migrate from the foetal liver or bone marrow to the thymus where they develop into mature T cells. The fate of the T cell during development is dependent on its differentiation status and the signals it receives as it interacts with thymic stromal cells and the environment. Thymocytes commit to cells expressing either the TCR$\alpha\beta$ or the TCR$\gamma\delta$, and develop into cells expressing either surface CD4 or CD8 molecules. The process of TCR gene rearrangement in the thymus may lead

to the development of T cells which recognise self antigen. Normally these autoreactive T cells are unresponsive to activation signals i.e. the cells are tolerant. Clonal deletion, clonal anergy and ignorance are considered to be the main possible ways in which tolerance occurs (Ramsdell and Fowlkes, 1990; Sprent *et al.*, 1990). Tolerance occurs in the thymus during T cell development (central tolerance) and in the periphery (peripheral tolerance).

1.2.1 Central tolerance

During thymic selection the T cell repertoire is shaped (Janeway, 1994). Positive selection selects for the maturation of immature CD4+ CD8+ thymocytes whose receptor is specific for a foreign peptide bound to self MHC (Blackman *et al.*, 1990; Ashton-Rickardt *et al.*, 1993). T cells which recognise the foreign peptide through their TCR but not the MHC molecule are deleted. Negative selection selects for CD4+ CD8- and CD4- CD8+ thymocytes recognising self peptides in the context of self MHC molecules with high affinity. These cells are deleted by apoptosis to eliminate potential self reactive T cell from the repertoire (Surch and Sprent, 1994). The overall aim of T cell selection in the thymus is clonal deletion of autoreactive T cells and the release of mature T cells into the periphery which do not recognise self peptides. However, autoreactive T cells are found in the periphery of healthy individuals. Since autoreactive diseases are relatively uncommon in the population it is now believed that peripheral tolerance is more important than central tolerance in controlling the responses by autoreactive T cells.

1.2.2 Peripheral tolerance

T cells specific for self proteins escape the thymic deletion process and exist in the periphery. This may be due to the self protein not being processed and presented in the thymus or being present in an amount too low to be detected by T cells. Liu *et al.* (1995) demonstrated that T cells specific for myelin basic protein (MBP) of low affinity escaped thymic deletion but would respond to high antigen concentrations of MBP in the draining lymph nodes. This indicates that thymic selection and deletion of autoreactive T cells is not complete.

Mechanisms within the periphery which prevent self reactive T cells from responding to self antigen include ignorance, deletion and anergy. Ignorance is a mechanism by which T cells do not respond due to either low levels of antigen or the affinity of the antigen receptor being too weak. Autoreactive T cells may be deleted from the periphery if they receive very strong signals from the APC on presentation of antigen. Lastly, anergy results from the absence of a costimulatory signal to the T cell which prevents the cell from proliferating and producing IL2 when restimulated with a specific antigen by a conventional APC (DeSilva *et al.*, 1991). Anergy is not static since specific signals are required to maintain this state. Reversal of anergy requires exposure to exogenous IL2 (Beverley *et al.*, 1992). Anergic cells are thought to maintain tolerance by down regulating immune responses and preserving the TCR repertoire (Taams *et al.*, 1999).

1.3 Autoimmunity

When the normal mechanical constraints which are present to protect against self - reactivity fail an immune response mounted against self protein may be initiated - 'autoimmune response'. Generally the mechanisms of tolerance are very effective in preventing serious unwanted effects but occasionally an autoimmune response may result in a clinical abnormality - 'autoimmune disease' (Sinha *et al.*, 1990). However, it is difficult to establish when the transition from an autoimmune state with no clinical manifestations to an autoimmune disease occurs. The mechanisms leading to the breakdown in tolerance and development of autoimmunity are poorly understood. However, since gross defects in tolerance would lead to overwhelming autoimmunity to an entire range of self antigens autoimmunity is more likely to be due to a limited dysregulation in peripheral and not thymic tolerance. Several critical events are likely to be involved (see section 1.3.1 - 1.3.8) but the integration of the collective information on the possible defects associated with autoimmune disease is difficult to interpret.

1.3.1 Incomplete deletion of self reactive clones

It is predicted that some T cells fail to be deleted in the thymus since they express a TCR that is of too low an affinity to bind self antigen, or that the self antigen is not expressed in the thymus. These autoreactive T cells are released into the periphery where they exist in a low frequency. Autoreactive T cell clones have been demonstrated in animal models, healthy individuals and patients. Kaliyaperumal *et al.* (1996) isolated T cells from mice recognising histone peptides. These peptides induced severe lupus nephritis when administered into the mice. Likewise, T cells to glutamic acid decarboxylase (GAD) have been reported in non - obese diabetic (NOD) mice (Kaufman *et al.*, 1993). Autoreactive T cells have been identified to myelin basic protein in multiple sclerosis patients and healthy individuals (Wucherpfennig *et al.*, 1994; Burns *et al.*, 1983) and in the experimental mouse model of the disease, experimental autoimmune encephalomyelitis (Gautam *et al.*, 1992; Dittel *et al.*, 1999). Likewise, autoreactive T cells specific for acetylcholine receptor have been isolated from Myasthenia Gravis patients and controls (Hohlfeld *et al.*, 1984). Furthermore, autoreactive cells specific to ribosomal P2 protein (Crow *et al.*, 1994), thyrotropin receptor (Mullins *et al.*, 1995) and topoisomerase I (Kuwana *et al.*, 1995) have been reported. Autoreactive T cells to other self proteins have been reported but determining which autoreactive T cells are primary to disease pathology and which arise as a consequence of the disease remains a challenging quest.

1.3.2 Molecular mimicry

For many autoimmune diseases the target autoantigen is unknown but when it is known it is unclear why it was selected. Much work is still being carried out to elucidate the initiating antigens although viruses and bacteria have been the favoured choice. An immune response to a non self protein which shares immunological epitopes with a self protein, and results in breaking tolerance to the self protein, is known as molecular mimicry. However, for an autoimmune disease to develop the immune response to self antigen must be sufficiently vigorous to perpetuate itself further by liberation of more self antigen.

Mamula *et al.* (1994) report that mouse snRNP injected into non autoimmune mice fails to induce an immune response. However, human snRNP stimulates anti - snRNP antibodies and specific T cells. Likewise, Dong *et al.* (1994) report that immunisation of BALB/c mice with both self p53 protein and Simian virus 40 large T antigen (SVTag) results in the production of autoantibodies to both SVTag and p53. Over time the autoantibodies to p53 fall off but on re-immunisation with self p53 protein alone these autoantibodies reappear. Immunisation with p53 alone did not result in autoimmunity.

Zhao *et al.* (1998) report T cells specific to corneal antigen cross react with herpes simplex virus - type I, and Bach *et al.* (1998) report T cells specific for GAD cross react with bacterial and viral antigens. Additionally, Gross *et al.* (1998) report T cells specific to the bacteria associated with the development of Lyme arthritis cross react with LFA-1 while Shimoda *et al.* (1995) report T cell crossreactivity between pyruvate dehydrogenase from humans and *Escherichia coli.* However, homology does not guarantee autoimmunity since the processing pathways are likely to be critical.

If molecular mimicry alone was responsible for autoimmunity surely it is more likely that we would see a greater percentage of the population with an autoimmune disease? Alternatively, molecular mimicry may be a common phenomenon but normal immunoregulatory mechanisms prevent injury from cross reactive responses. It is only those individuals in which the mechanisms fail who develop an autoimmune disease.

1.3.3 Inappropriate antigen expression

A mechanism for accessing self antigens which are not normally seen by the immune system ('cryptic epitopes') is fundamental in creating opportunities for the development of autoimmune disease. Such epitopes have been shown to elicit strong immune responses although this could depend on the efficiency of the APC to present the peptides (Mamula, 1993). The response to a cryptic epitope may overcome tolerance to a self protein since immunisation with self cytochrome c protein in the presence of a cryptic cytochrome c peptide results in a T cell response to the self cytochrome c protein (Mamula, 1993).

A possible mechanism for revealing cryptic epitopes is abnormal processing of the antigen. Sjostrom *et al.* (1998) showed that T cell responses to gliadin peptides is improved if the peptides are modified by deamidation. Corthay *et al.* (1998) show that glycosylation is critical for the recognition of type II collagen. Alternatively, proteins may be specifically cleaved (U1-70K protein and type I keratins) or phosphorylated during apoptosis to reveal cryptic epitopes (Casciola-Rosen *et al.*, 1994b; Ku *et al.*, 1997; Utz *et al.*, 1997). Likewise, the release of sequestered antigen by infection, inflammation and hence tissue damage may initiate autoimmunity. Local tissue damage leads to an increase in peptide presentation of both the foreign and self peptide in the draining lymph node. This is simultaneous with an increase in the costimulation signals generated through the non - self immune response. Together these signals may be sufficient to activate a response in the autoreactive T cell population.

1.3.4 Epitope spreading

The mechanism whereby an immunological response to one antigen initiates a response to another antigen is termed 'epitope spreading'. In the MRL-*lpr* mouse model of systemic lupus erythematosus (SLE) the initial antibody is directed against the snRNP protein A. As the disease develops antibodies to snRNP 70K, A, B and D proteins arise (Fatenejad *et al.*, 1994). Additionally, Reynolds *et al.* (1996) and Topfer *et al.* (1995) report that mice immunised with human La, a candidate T cell autoantigen in SLE, stimulates antibody production directed against La. The antibody response spreads to multiple epitopes within the La protein and later to the Ro protein. Likewise, immunisation with Ro protein leads to the generation of antibodies to Ro and La (Scofield *et al.*, 1996). However, Reynolds *et al.* (1996) showed that mice immunised with mouse La did not provoke an immune response to the antigen. Similarly, in the NOD mice model, early disease is limited to immune responses to glutamic acid decarboxylase, but as the disease progresses the T cell response spreads to involve other proteins (Kaufman *et al.*, 1993). Likewise, Lehmann *et al.* (1992) showed that in the animal model of multiple sclerosis the immune response is initially confined to a single determinant of myelin basic protein (MBP) Ac1-11, but as the disease develops the T cell response diversifies to incorporate cryptic self peptides of MBP.

However, despite such studies T cell lines derived from patients with multiple sclerosis have been shown to be restricted to immunodominant MBP peptides (Wucherpfennig *et al.*, 1994).

1.3.5 Linked recognition

Linked recognition is a term used to describe an immune response in which a response is initiated to a single antigen within a complex but leads to an immune response to other antigens within the complex. The protein to which the initial immune response occurs is often described as the hapten carrier and is required for an immune response to be generated against other peptides in the complex (Martin and Weltzien, 1994). Steinhoff *et al.* (1994) demonstrated an antibody response to vesicular stomatitis virus (VZV) in VZV transgenic mice only when the virus was coupled to sperm whale myoglobulin. Thus, self reactive B cells could be triggered if they receive T cell help from a linked antigen. In systemic lupus erythematosus it has been proposed that the autoantigens responsible for the initiation of the disease are located in apoptotic blebs co-concentrated with viral antigens (Rosen *et al.*, 1995).

1.3.6 Bystander effect

The presence of serum autoantibodies indicates that autoreactive B cells exist. Normally autoantibodies are of the IgM isotype and are not harmful, but in autoimmune diseases these autoantibodies are class switched, are of high affinity and show somatic hypermutations. This suggests that interaction of a specific autoreactive B cell with a specific T cell was involved in the response. However, very few autoreactive T cells with specificity which parallels the antibody response have been described. It has therefore been proposed that B cells are activated by cytokines, including IL12, TNFα and IFNγ, secreted by adjacent non specific immune cells. This mechanism utilising non specific T cell help is known as a bystander effect and has been suggested to aid autoimmunity (Tough *et al.*, 1996). Limited evidence is available for this mechanism but Horwitz *et al.*

(1998) clearly demonstrate that Coxsackie virus contributes to diabetes in NOD mice through bystander damage and not molecular mimicry.

1.3.7 Altered expression of surface molecules

Aberrant expression of the MHC, adhesion and costimulatory molecules on cells which do not normally express them may initiate an autoimmune response. Simon *et al.* (1991) report that PMA treated keratinocytes can induce cell proliferation due to an upregulation of ICAM-1 (CD54) expression. Likewise, cells expressing the costimulatory molecule B7 (CD80) may activate T cells without the need for peptide recognition (Mamula, 1995). However, Williams *et al.* (1994) show that keratinocytes expressing B7 are unable to trigger inflammatory changes although they can amplify a response to antigens presented by B7 expressing cells. Alternatively, the release of sequestered antigen, altered distribution of an autoantigen or altered expression of an antigen may influence the outcome of an immune response (Kawahata *et al.*, 1999; Grolz and Bachmann, 1997)

1.3.8 Regulatory T cells

Regulatory T cells including suppressor cells may be important in controlling and preventing the immune system responding to self antigen (Mason and Powrie, 1998). If regulatory T cells confer protection from autoimmunity anergy of these cells may mediate breakdown of tolerance and increase susceptibility to autoimmune disease. Therefore anergy of Th1 cells which mediate autoimmunity may be beneficial and Th2 anergy harmful. In mice which develop IDDM no IL4 is produced suggesting that anergy of Th2 cells mediates the onset of disease. However, treatment with IL4 prevents onset of IDDM. The existence of regulatory cells is debatable due to the difficulty in isolating these cells, but Kumar and Sercarz (1993) report that mice recovering from MBP induced EAE produce regulatory T cells to down regulate the proliferative response to the initiating autoantigen. Furthermore, Seddon and Mason (1999) report the generation of regulatory cells in autoimmune thyroiditis only when the autoantigen is present.

1.4 Systemic Lupus Erythematosus

This section introduces the clinical and immunological features of the autoimmune disease, systemic lupus erythematosus (SLE). The disease is characterised by the production of antibodies to double - stranded DNA. However, serum antibodies to nuclear proteins including La, Ro, Sm and Jo-1 are found in some patients suggesting that these proteins are potential T cell autoantigens. The autoantibodies found in SLE patients are of high affinity and show somatic hypermutations with class switching to the IgG isotype. The disease is also HLA restricted which further suggests that a CD4 T cell dependent mechanism is involved in the generation of autoantibodies.

1.4.1 Clinical features of Systemic Lupus Erythematosus

Systemic lupus erythematosus is a prototypic systemic autoimmune disease involving multiple organ systems. As reviewed by Kotzin (1996) it predominantly affects females, particularly women of child-bearing age (the female to male ratio of 9:1 is greater in the 15 - 50 year old range) and is 2 - 4 times more likely in ethnic populations (Johnson *et al.*, 1995). In males, SLE is more common in those under 16 years of age. The overall prevalence is similar to multiple sclerosis, approximately 1 in 2000. The clinical symptoms are diverse and variable with unpredictable exacerbations and remissions. The disease manifests itself primarily in the form of skin rashes, photosensitivity to sunlight and joint pain. Fatigue is also common amongst patients and is associated with disease activity (Petri, 1995). As the disease progresses patients develop severe kidney impairment due to the deposition of immune complexes composed mainly of cationic anti - DNA antibodies, lymphopenia and musculoskeletal damage (Kotzin, 1996). Pulmonary haemorrhage is rare but cardiac complications including rhythm disturbances can occur. Lymphocytic vasculitis, cervical atypia, neurophysiological abnormalities and nephritis develop with age. Disease severity is dependent on patient care, psychosocial factors, the age of disease onset and the duration of the disease. Karlson *et al.* (1997) showed that education, counselling and extensive medical care all improve the outcome of the disease. Medical

treatment normally involves administration of steroids and cytotoxic drugs including cyclophosphamide.

1.4.2 Etiology of Systemic Lupus Erythematosus

The etiology of SLE remains elusive but there is some association with environmental and genetic factors. An association between hormonal levels and SLE has been suggested due to the disease prevalence in the female population and to post pubertal SLE children who have increased levels of luteinizing hormone, follicle stimulating hormone and prolactin. Plasma levels of oestrogen in SLE has been shown to be normal but administration of exogenous hormones including oestrogen have been shown to alter the disease outcome (Lahita, 1992).

Viral and bacterial infections, including human immunodeficiency virus (HIV), human T cell leukemia virus (HTLV), cytomegalovirus (CMV) and Epstein - Barr virus (EBV) are associated with the disease (Herrmann *et al.*, 1996; Nakamura *et al.*, 1998; Incaprera *et al.*, 1998). The role of the viruses is unclear although EBV and CMV are reported to cause redistribution of the autoantigen La (Baboonian *et al.*, 1989). A genetic predisposition in terms of HLA haplotype and an early deficiency in complement activation, important in inhibiting immune complex formation and solubilizing existing complexes, are also implicated in the disease.

1.4.3 Autoantibodies associated with SLE and other autoimmune diseases

Little is known about the immune response which leads to the production of autoantibodies associated with systemic lupus erythematosus, a disease characterised by B cell hyperactivity and the generation of immune complexes. A common denominator is the presence of serum IgG anti - dsDNA antibodies which are present in 50 - 75% of patients with SLE (Kotzin, 1996; Tomer *et al.*, 1993). A sudden change in these antibody levels often indicates a disease flare. Anti - dsDNA antibodies are involved in end organ damage through deposition in the kidneys but may not be involved in the initiation of the disease.

Other characteristic polyclonal autoantibodies found in sera from patients with SLE are against 1) nuclear proteins including histones, chromatin, U1 and nucleosomes, 2) extractable nuclear proteins including Sm, RNP, Jo-1, Ro and La and 3) negatively charged phospholipids including cardiolipin, phosphatidylserine, phosphatidic acid and phosphatidylethanolamine (Tomer *et al.*, 1993; Elkon, 1995; Drouvalakis and Buchanan, 1998). No clear association between antibody titer and disease activity has been established nor why these particular antigens are selected as targets in the disease. However, the concentration of nucleosomes in the plasma of patients with SLE is much higher than that found in healthy individuals (Amoura *et al.*, 1997). This may explain why nucleosome autoantibodies are detected early in the disease.

In SLE the incidence of serum autoantibodies to Ro is 17 - 63% and to La is 6 - 35% depending on the detection method used to measure the antibody titres (Tomer *et al.*, 1993). Some SLE patients with anti - Ro antibodies never develop anti - La antibodies, but it is rare to find serum anti - La antibodies without the presence of anti - Ro antibodies. Anti - Ro antibodies are associated with an increased susceptibility to UVA and photosensitivity while the presence of serum anti - La autoantibodies often indicates a milder form of the disease with lower anti - dsDNA antibody titres and less nephritis. The presence of serum anti - La and anti - Ro antibodies is associated with the HLA DR3 DQ2 haplotype, while anti - Ro antibodies alone are associated with the HLA DR2 DQ6 haplotype (Reveille *et al.*, 1991; Hamilton *et al.*, 1988).

Autoantibodies to Ro and La have been implicated in another autoimmune disease, Sjogren's syndrome (SS) (Harley *et al.*, 1986). This disease is an organ specific autoimmune disease which manifests itself primarily in females as a chronic inflammation of the salivary and lacrimal glands (Fox, 1995). The patients have dry eyes, dry mouth and an autoimmune arthritis. The destruction of the salivary glands is observed in the more serious cases. Prognosis is good although a small number of patients who have hypergammaglobulinaemia develop B cell lymphomas.

Anti - Ro autoantibodies have also been found in 58% of patients with myositis but studies

have also shown these antibodies, as well as anti - La autoantibodies, to be present in healthy individuals following B cell activation *in vitro* (Venables *et al.*, 1988). It is unclear why SLE, SS and myositis patients develop autoantibodies to the same antigens since the diseases are very different, despite the antigens being ubiquitous intracellular proteins. The autoantibodies may be characteristic of the diseases, but it is unknown what role they play in the disease pathogenesis.

Vascular complications, recurrent foetal loss and thrombocytopenia described in SLE are associated with serum antibodies to phospholipids, including cardiolipin, phosphatidyl - serine, phosphatidylinositol and phosphatidylethanolamine (Rauh and Janoff, 1992). However, a group of patients have been described who have these anti - phospholipid antibodies but do not fulfil the ARC criteria for SLE. This disease is known as primary anti - phospholipid syndrome (APS) (Roubey, 1996). In both SLE and APS, anti - cardiolipin antibodies are used to clinically diagnose vascular complications. Recent studies have revealed that the β2 glycoprotein 1 plasma protein (β2GP1) is a necessary cofactor for the binding of anticardiolipin antibodies to cardiolipin (Roubey *et al.*, 1995). Additionally, β2GP1 - dependent anticardiolipin antibodies are associated with autoimmune disease (Guerin *et al.*, 1997; Cabiedes *et al.*, 1995).

1.4.4 Autoantigen features

La, also referred to as SSB, is a 48kDa (408 amino acids) protein which is mainly located in the nucleus but is translocated to the cytoplasm under conditions of stress (Chambers *et al.*, 1988; Bachmann *et al.*, 1986). It is susceptible to degradation by proteases including papain, trypsin and *Staphylococcus aureus* V8 protease although two domains remain relatively protease resistant (Chan *et al.*, 1986). Several degradation products have been reported including a 43kDa protein, a 40kDa protein (which is considered to be the major breakdown product) and a 28kDa protein (Chan *et al.*, 1986). However, Troster *et al.* (1994) have also demonstrated a second site for mRNA transcription for La suggesting two different protein products of different sizes. The role of La is unclear but it appears that the association of La with newly synthesised RNA polymerase III transcribed RNA

molecules, including tRNA, RoRNA and ssrRNA, leads to the termination of transcription (Rinke and Steitz, 1982). La has also been shown to promote the translation of viral mRNA, including poliovirus, and to bind and unwind RNA (Meerovitch *et al.*, 1993).

La associates with human cytoplasmic RNA's but only in the presence of the Ro polypeptide (Mamula *et al.*, 1989). Ro protein, also referred to as SSA, exists as three forms encoded for by separate gene segments; Ro46 (46kDa), Ro52 (52kDa) and Ro60 (60kDa) (McCauliffe and Sontheimer, 1993). Ro46 is suggested to be retained within the reticulum and is involved in protein assembly. Ro52 is found predominantly in the nucleus and may act as a gene regulator molecule. Studies have shown that Ro52 is not a stable component of the active ribonucleoprotein (RNP) complex and that only a small percentage of the total Ro52 binds to the RNP complex (Pruijn *et al.*, 1991). Ro60 is expressed in the RNP complex which is exclusively localised in the cytoplasm but is displaced to the cell surface by CMV.

The antigenic properties of Ro and La appear to be independent of an association with RNA although the structural association of Ro60 and La may explain the co - existence of anti - Ro60 and anti - La autoantibodies. Immunisation of mice with recombinant Ro60 leads to the generation of anti-La antibodies as well as anti - Ro60 antibodies, and vice versa, but these mice do not develop symptoms associated with SLE (Chambers *et al.*, 1988; Topfer *et al.*, 1995; Scofield *et al.*, 1996). The initial antibody response is aimed at specific sites of either the La or Ro protein but as the disease develops the antibody response diversifies and spreads through the proteins. However, anti - Ro antibodies may exist in patients with SLE without anti - La antibodies. This suggests that an initial monospecific anti - Ro antibody response is insufficient to recruit immunity to La and hence break tolerance to La (Gordon *et al.*, 1994).

1.4.5 T cell responses

The pathology of SLE in so many tissues suggests a general failure of self tolerance. A wealth of T and B cell abnormalities have been described but, in general, antibody

production is hyperactive while T cells show lymphopenia. There is a reduced number of CD4 and CD8 T cells (Bakke *et al.*, 1983; Morimoto *et al.*, 1982). The lymphopenia correlates with anti - lymphocyte antibodies, cell cycle, medication and disease activity (Tokano *et al.*, 1997; Winfield and Mimura, 1992). Within the CD4 and CD8 T cell subsets, CD28+ cells are decreased (Alvarado *et al.*, 1994). Kaneko *et al.* (1996) report that this preferential differentiation is due to increased apoptosis. Additionally, there is an increased population of CD4- CD8- TCR$\gamma\delta$ cells (Gerli *et al.*, 1991). This phenotype is associated with increasing age which suggests early usage of regenerating T cells through extrathymic differentiation of bone marrow progenitor cells in the gut and liver. The proportion of T cells expressing CD45RA is also reduced while CD45RO expression is increased (Sato *et al.*, 1987). Antibodies to the different isoforms of CD45 have been identified in SLE which may favour the cell skewing (Mimura *et al.*, 1990). However, more recently, Gordon *et al.* (1996) report that disease flare is associated with CD45RA and CD45RB expression suggesting that resting T cells are activated in favour of recruitment of existing memory T cells.

Despite the reduced absolute number of T cells in SLE, the cells are often in an activated state as shown by the increase in surface IL2 receptor and HLA DR expression (Chan *et al.*, 1996; Tsuchiya *et al.*, 1988). However, *in vitro* T cells from patients with SLE show reduced responses to anti - CD2 monoclonal antibodies (Fox *et al.*, 1991; Horwitz *et al.*, 1997) and to PHA (Murakawa and Sakane, 1988; Utsinger and Yount, 1977). In contrast, the proliferative response to anti - CD3 monoclonal antibody is enhanced (Stekman *et al.*, 1991). However, the response to tetanus toxoid and PPD, which use the CD3 pathway to signal into the cell, are reduced (Gottlieb *et al.*, 1979; Fox *et al.*, 1991; Tsokos *et al.*, 1996). The addition of PMA (Blasini *et al.*, 1994), anti - CD28 (Horwitz *et al.*, 1997; Alvarado *et al.*, 1994) or depletion of macrophages (Fox *et al.*, 1991) enhances the proliferative response to antigens. In SLE a reduced mixed lymphocyte reaction (MLR) is also reported compared to responses obtained in healthy individuals (Sakane *et al.*, 1978). Sera from patients with SLE are reported to suppress the MLR in healthy individuals (Hahn *et al.*, 1982). The most likely explanation is the presence of anti - lymphocyte antibodies in the sera (Williams *et al.*, 1973).

The highly activated phenotype associated with T cells from SLE patients but the poor *in vitro* response suggests that T cells are exhausted. At present it is unclear whether this is due to an intrinsic T cell disorder, a result of poor communication between the T cell and APC, or a faulty APC function. However, autoreactive T cells to ribosomal P protein, U1RNP and nucleosomes have been isolated from peripheral blood taken from patients with SLE and healthy individuals (Crow *et al.*, 1994; Okuba *et al.*, 1995; Lu *et al.*, 1999). La and Ro specific T cells have been demonstrated in peripheral blood taken from SLE patients and healthy individuals although there was no difference in the number of subjects responding (Helsloot and Sturgess, 1997; Halse *et al.*, 1996). These reports indicate that autoreactive T cells have escaped deletion but their relevance in the disease is unknown.

1.4.6 Cell signalling defects.

The inability to phosphorylate factors necessary to convey signals from the cytosol to the nucleus could modify a T cell response to a stimuli or interfere with the switching of a Th0 cell to a Th1 or Th2 type cell (Weiss and Littman, 1994). Critical events for signalling into the cell includes correct binding of the antigen to the receptor, tyrosine kinase phosphorylation, activation of protein kinase C and increases in intracellular calcium. In SLE there is no evidence to suggest that the autoantigen binds incorrectly to the TCR preventing signals from being correctly generated within the T cell. However, a reduction in protein kinase C (pKC) activity in T cells from SLE patients has been reported, but the range of pKC isotypes affected has not been studied (Tada *et al.*, 1991). Abnormal intracellular calcium levels have also been reported (Vassilopoulos *et al.*, 1995) as well as reduced protein kinase A (pKA) isozyme type I activity which mediates cell maturation (Kammer *et al.*, 1994). CD4 T cells from patients with SLE show a suppressed level of cAMP following adenosine treatment compared to T cells from healthy individuals (Mandler *et al.*, 1982). These biochemical and signalling defects could therefore be an underlying factor in T cell activation reported in SLE.

1.4.7 Cytokine defects

Cytokines play an important role in the maturation of the immune response to stimuli. Hence, any defect in the production of cytokines may upset the balance in the cellular response. However, cytokines act locally therefore serum levels may not reflect the response in lymphoid tissue. In SLE serum levels of IL6, IFNγ and IL10 are increased while IL2, TNFα and IL12 are reduced (Hagiwara *et al.*, 1996; Llorente *et al.*, 1994; Tsokos *et al.*, 1986). Levels of IL10 have been shown to correlate with the disease activity of SLE (Park *et al.*, 1998).

The spontaneous production of IL2 is low and a decreased IL2 production is observed in mitogen stimulated PBMC's (Linker-Israeli *et al.*, 1983; Alcocer-Varela and Alarcon-Segovia, 1982). This may indicate an intrinsic defect in CD4+ T cells, the major producers of IL2, or may simply be an *in vitro* artifact. However, the production of IL2 may be enhanced with costimulation from mitogen, depletion of macrophages or resting the cells prior to stimulation (Warrington *et al.*, 1989; Murakawa and Sakane, 1988; Huang *et al.*, 1986). IL1 enhances IL2 production following mitogen stimulation of PBMC's in healthy individuals but not in SLE patients suggesting an underlying defect in the IL1 receptor of SLE patients. Spontaneous and mitogen - induced secretion of IFNγ and IL12 is decreased while IL6 and IL10 is increased (Hagiwara *et al.*, 1996; Linker-Israeli *et al.*, 1991). However, IL4 levels are reported to be unchanged in patients with SLE but this cytokine is associated with allergic responses (Linker-Israeli *et al.*, 1991; Fujihasi *et al.*, 1993). A reduction in TNFα in response to a lectin was reported to be associated with the HLA DR2/DQw1 haplotype (Jacob *et al.*, 1990). TNFα and MHC genes are located on the same chromosome suggesting a defect in chromosome 6 transcription.

1.4.8 Antigen presenting cells

A fundamental role for APC's is to process and present antigen in the context of the MHC molecule to T cells. The reduced T cell response to recall antigens in patients with SLE suggests a defect in the APC and not in the T cell since processing of the antigen is

required (Tsokos *et al.*, 1996). However, some patients with SLE show a poor T cell proliferative response to both recall antigens and abnormal responses to alloantigens, indicating defects occur in both the APC and T cell (Via *et al.*, 1993). The depletion of macrophages from PBMC's improves the stimulatory response to antigen further supporting the role of defective APC's in SLE.

A defect in the FcγR function has been observed in SLE suggesting an inability to take up exogenous antigen bound to antibody and to activate the complement cascade. Frank *et al.* (1979) report that cells from SLE patients had a decreased ability to clear IgG tagged red blood cells. However, Mamula *et al.* (1994) report that B cells are capable of presenting lupus autoantigens which initiate autoreactive T cell responses. Additionally, the processing and post - translational modifications of an autoantigen may influence the T cell response. Sjostrom *et al.* (1998) report that deamidation of gliadin, a T cell autoantigen in coeliac disease, enhances the proliferative response to the antigen. Likewise, the glycosylation status of the autoantigen may have similar effects (Corthay *et al.*, 1998). However, such mechanisms have not been reported in SLE. Furthermore, the increased expression of surface MHC molecules in patients with SLE suggests enhanced antigen presentation. Amino acid and conformational changes in the MHC binding groove may affect antigen presentation by altering the anchor sites for the peptide and TCR. Reveille *et al.* (1991) report that anti - Ro and anti - La antibodies are more closely linked with the HLA DQβ1 allele having a leucine at position 26 of the outer most domain of the molecule. Since this residue maps to the floor of the class II binding cleft it possibly plays a preferential role in the presentation of peptides.

Another primary function of APC's is to deliver accessory signals either through the production of cytokines or through cognate interactions with T cells. APC's from patients with SLE have a reduced IL1 production upon stimulation *in vitro* which may influence the production of IL2 by T cells. However, since cytokines have multiple roles it is difficult to unravel their relevance in disease. An essential costimulatory molecule present on activated APC's is B7, which interacts with CD28 molecules constitutively expressed on T cells (Manickasingham *et al.*, 1998; Damle *et al.*, 1991). This interaction can

reestablish a response in anergic T cells and also prevent cells entering an anergic state. Tsokos *et al.* (1996) report that CD80 (B7.1) expression is not upregulated on APC's from SLE patients in response to IFNγ compared to healthy individuals and may therefore account for the APC abnormality. However, Takasaki *et al.* (1998) report that the expression of CD80 on PBMC's from patients with SLE is much higher than that found on those from healthy individuals. This suggests that APC's within the blood of SLE patients are already highly activated which may lead to inappropriate signalling into the APC (Garcia-Cozar *et al.*, 1996). Additionally, anti - CD86 but not anti - CD80 monoclonal antibody have been reported to inhibit the production of autoantibodies (Nakajima *et al.*, 1995). UV light, to which SLE patients are sensitive, has been shown to affect the capacity of APC's to deliver costimulatory signals (Kitajima *et al.*, 1996). Kitajima *et al.* (1996) suggest that apoptosis of APC's following UV sensitisation could deliver unusual activation signals to the T cell which may lead to cell unresponsiveness.

1.4.9 Apoptosis and SLE

Apoptosis is a form of controlled cell death in which the cell undergoes DNA fragmentation followed by nuclear fragmentation (Cohen, 1993). The vesicles formed are known as apoptotic blebs or bodies. During apoptosis, proteins which are specifically cleaved or phosphorylated have been reported to be targets for autoantibodies (Utz *et al.*, 1997). Casciola-Rosen *et al.* (1994b) reported that U1-70kDa protein is specifically cleaved during apoptosis while Rutjes *et al.* (1999) reported that La protein is cleaved and dephosphorylated during apoptosis. RoRNP, U1-70kDa, La, Ro and nucleosomes are found within apoptotic blebs or on the surface of the blebs (Casciola-Rosen *et al.*, 1994a). In respect of Ro and La two types of apoptotic blebs have been reported; apoptotic blebs containing only Ro and apoptotic blebs containing Ro and La (Casciola - Rosen *et al.*, 1994a). This may explain why anti - Ro antibodies occur in the absence of anti - La antibodies but anti - La antibodies do not occur without anti - Ro antibodies. The presence of serum anti - Ro antibodies are associated with renal disease whilst the presence of serum anti - Ro and anti - La antibodies is associated with SS and SLE. This therefore suggests La plays a role in the pathogenesis of the autoimmune diseases, SS and SLE.

Apoptosis is a major influence concerning the pathogenesis of SLE. In addition to the role of La in SLE and SS β_2 glycoprotein 1 has also been implicated in disease pathogenesis. Alarcon-Segovia *et al.* (1997) showed that some anti - phospholipid antibodies require the presence of β_2 glycoprotein 1 for the binding to phospholipids. Manfredi *et al.* (1998a and 1998b) showed that anti - phospholipid antibodies bind apoptotic cell membranes in the presence of β_2 glycoprotein 1 which leads to increased phagocytosis. It has therefore been suggested that the clearance of apoptotic cells occurs via a β_2 glycoprotein 1 dependent pathway. However, in SLE the clearance of apoptotic material is defective which may be due to a malfunction in the macrophages, complement deficiency or the presence of serum anti - β_2 glycoprotein 1 antibodies (Herrmann *et al.*, 1998). Apoptotic blebs which are not removed become lysed releasing their contents into the periphery. This may therefore provide a source of antigen to which antibodies may be generated (Mevorach *et al.*, 1998).

Bcl-2 is a protein which prolongs cell survival when it is expressed. Bcl-2 transgenic mice show lupus - like characteristics suggesting a role for bcl-2 in the disease (Strasser *et al.*, 1991). CD3 T cells and PBMC's from SLE patients show an increased expression of bcl-2 which may prolong the survival of these cells (Rose *et al.*, 1997). Ohsako *et al.* (1994) report that the expression of bcl-2 is associated with disease severity. However, anti - CD3 monoclonal antibodies increase the rate of apoptosis in $CD28^+$ T cells suggesting other factors influence cell death (Kaneko *et al.*, 1996). Melanocytes also have an increased expression of bcl-2 and are therefore protected from apoptosis. However, the Afro - Caribbean and black populations have an increased number of melanocytes but also show an increased risk of SLE suggesting apoptosis is insufficient to cause the disease. In contrast to bcl-2, PBMC's from patients with SLE show increased expression of Fas, a protein which signals the cell to die by apoptosis (Mysler *et al.*, 1994).

1.5 Experimental models of SLE

The discovery of murine strains which spontaneously develop an autoimmune syndrome with similar features as human SLE provides a valuable experimental system for analysing genetic contributions and cellular mechanisms involved in the disease pathogenesis. There are three murine models; (NZBxNZW)F1, MRL-lpr and BXSB, all of which develop anti - nuclear antibodies, immune complex mediated renal disease, which resembles human lupus nephritis observed in SLE patients, and thrombosis. BXSB and (NZBxNZW)F1 strains more closely resemble human lupus compared to MRL-lpr mice with respect to the multigenic basis for the disease. However, all models suggest ubiquitous pathogens are involved in the disease since multiple autoantibody responses are found (Steinberg *et al.*, 1984).

1.5.1 (NZBxNZW)F1

Of the three experimental models for lupus this mouse strain, (NZBxNZW)F1, has been studied most extensively (Steinberg *et al.*, 1981). All affected animals are female and develop renal failure secondary to immune complex deposition in the kidneys. Neither New Zealand black (NZB) or New Zealand white (NZW) mice are prone to lupus-like syndromes. There is no common genetic defect which predisposes autoimmunity in New Zealand mice (Drake *et al.*, 1995). MHC genes contribute to the disease, since the class II β chain mutations of the NZB strain (NZB H-2^{bm12}) increases autoantibody production in the (NZBxNZW)F1 strain. The disease can be transferred by haemopoietic stem cells since bone marrow of NZB mice induces tolerance defects in irradiated recipients and gives rise to B cells which produce increased amounts of autoantibodies (Laskin *et al.*, 1982).

(NZBxNZW)F1 mice produce a spectrum of autoantibodies including antibodies to ssDNA, dsDNA and histones. They do not produce anti - phospholipid antibodies. The autoantibodies appear to be responsible for tissue damage and not for the initiation of the disease since young mice secrete predominantly IgM antibodies. This strain of mice use

crossreactive IgG secreting B cells, a similar feature found in SLE patients where 10 - 18% IgG anti - DNA producing B cells are crossreactive (Pisetsky, 1998). $CD4^-CD8^-TCR\alpha\beta^+$ T cells are predominantly found in the model with a specific expansion of T cells expressing the Vβ8 TCR (Kumar *et al.*, 1989). Increased levels of ICAM-1 are also found on glomeruli suggesting a role for adhesion molecules in the development of disease. Mice immunised with anti - CD4, TNFα, IFNγ or LFA-1 antibodies are protected from developing the disease while co-administration of anti - CD80 and anti - CD86 monoclonal antibodies inhibits disease progression (Nakajima *et al.*, 1995). The mice have increased levels of Th2 type cytokines including IL-5 and IL-10 (Chen *et al.*, 1997).

1.5.2 MRL - lpr mice

This congenic strain of mouse has the *lpr* (lymphoproliferation) gene inbred which arises due to the integration of a retroviral gene and hence a mutation into the Fas molecule (Cohen and Eisenberg, 1991). MRL-lpr mice are prone to a lupus like syndrome with the single gene defect accelerating the disease rate. If the *lpr* gene is transferred into a non-SLE mouse the autoimmune disease still manifests itself. The *lpr* gene defect is thought to mainly interfere with mechanisms of peripheral and not thymic tolerance resulting in the retention of some self reactive cells. The disease is not sex-related and shows a multi step development with symptoms including splenomegaly, lymphadenopathy, arthritis, vasculitis, hypergammaglobulinaemia and early mortality. They also develop lymphocytic infiltrations of the submandibular glands so this strain is a good model for Sjogren's syndrome.

A wide spectrum of autoantibodies, including anti - phospholipid antibodies' are produced. MRL-lpr mice have anti - La autoantibodies but they show a different specificity for synthetic La peptides compared to human anti - La antibodies (Tomer *et al.*, 1993) The B cells respond normally to stimuli and it has been suggested that proliferating T cells preferentially release cytokines which stimulate B cell growth and differentiation. There is a preferential activation of IFNγ secreting cells and a reduction in IL-4 secreting cells.

There is also an increase in serum IL-1β and TNF levels. This altered cytokine balance may affect the maturation of T cells since there is an increased number of immature T cells which show a poor proliferative response to stimuli. In order to stimulate proliferation in T cells of MRL-lpr mice with PMA and anti - CD3 antibodies, anti - CD28 antibody co-stimulation is required (Dayal and Kammer, 1996).

1.5.3 BXSB strain

The BXSB strain, a cross between C57BL/6 (B6) female mice with SB/le male mice, have a genetic mutation on the Y chromosome which is known as the Yaa gene (Y chromosome linked autoimmune acceleration) (Izui *et al.*, 1995). Affected mice are therefore male. The Yaa gene is unable to promote lupus-like autoimmunity since the Yaa gene in non prone SLE mice (B6) does not induce lupus. Autosomal genes, for example MHC genes, are therefore likely to be involved. BXSB mice show early mortality and die within 4 - 6 months. At 4 months they show increased levels of anti - dsDNA IgG2a, IgG3 antibodies and CD4 Th cells. Murine IgG2a is associated with complement activation. T cells express an activated memory phenotype. Resting B cells from these mice show hyperproliferation in response to PMA/ionomycin, LPS, anti - IgM or CD40L. T cells show reduced proliferation, IL-2 secretion and IFN$_\gamma$ production in response to anti - CD3 antibody stimulation.

1.6 Aims of this study

T cells play a pivotal role in the immune response including in the generation of antibodies. Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterised by the production of excess autoantibodies to DNA and nucleoproteins including La, Ro and Sm. Accumulating evidence has implicated T cells in the pathogenesis of SLE; antibodies are of high affinity, class switched and show somatic hypermutations and the disease is associated with the HLA DR3 DQ2 haplotype. These immunological features and the characteristic flares of SLE, therefore, indicate that the disease is driven by self proteins in a T cell dependent manner. Obvious antigens to be driving the disease are those to which serum antibodies are found and include La and Ro proteins. A demonstration of the presence of T cells specific for these antigens would provide clues as to the pathogenesis of systemic lupus erythematosus and increase our understanding of other autoimmune diseases.

There is a general consensus that PBMC's from SLE patients show a reduced proliferative response to antigens *in vitro*. It has also been suggested that these responses can be normalised by altering culture conditions. Since the primary aim was to examine T cells in the disease an initial aim was to establish a T cell proliferative assay using PBMC's from SLE patients.

SLE patients with APS have antibodies to cardiolipin, a phospholipid present in serum. These antibodies are used in the diagnosis of the disease. However, it is not clear how antibodies to a lipid arise. One theory suggests that lipids are bound to proteins and it is the protein element of the complex which is responsible for the initiation of the response to the lipid. It has been shown that cardiolipin is bound to a protein cofactor, β_2 glycoprotein 1, which is important in the binding of cardiolipin to anticardiolipin antibodies. The aim was therefore to determine whether T cells to the β_2 glycoprotein 1 cofactor occur in SLE and if the proliferative response was associated with the presence of serum autoantibodies.

Understanding the mechanism underlying T cell responses to autoantigen is pivotal to the understanding of the development of autoimmunity. Since anti - La antibodies are implicated in the pathogenesis of the disease the La protein was used as a candidate T cell autoantigen to study T cell responses in SLE. La specific T cells recognising recombinant forms of the protein have previously been demonstrated (Helsloot and Sturgess, 1997; Halse *et al.*, 1996). However, the frequency of responding individuals did not differ between healthy individuals and patients. The issue of whether T cells were responding to contaminating *E. coli* antigens in the recombinant preparation or that new epitopes are revealed by altered antigen processing were not studied. La peptides were therefore used in the present study as an approach to bypass the issue of antigen processing differences which may occur in the diseased state.

Chapter 2

Materials

2.1 Tissue Culture Plastics

1ml cryovials	Life Technologies, Renfrewshire, England
25cm and 175cm flasks	Life Technologies, Renfrewshire, England
25ml universals	Philip Harris, Wilford, England
50ml conical 'Falcon' tubes	Life Technologies, Renfrewshire, England
96 x 200ml well, flat bottom	Life Technologies, Renfrewshire, England
Acrodisc filters (0.2μm)	Gelman Science Ltd., Northampton, England
Pipettes (1ml - 25ml)	Farenheit Lab Supplies, Nottingham, England
Syringes (1 - 50ml)	Appleton Woods, Birmingham, England

2.2 Tissue Culture Media and Reagents

Anti - CD28 monoclonal antibody	Becton Dickinson, Oxnard, California, USA
Beta Plate scintillation fluid	LKB Scintillation Products, England
BCA kit	Pierce, Illinois, USA
Dimethylsulphoxide	Sigma, Poole, England
DMEM	GibcoBRL, Life Technologies Ltd, Scotland
FACS tubes	Falcon, New Jersey
Ficoll - Paque	Pharmacia Biotechnology Ltd, Milton Keynes, UK
Foetal calf serum	Sigma, Poole, England
Glutamine	Sigma, Poole, England
Goat anti - mouse IgG Dynalbeads	Dynal UK, Merseyside, England
Hepes Buffer	Sigma, Poole, England
Human β_2 glycoprotein 1	Serbio or IDRL, Birmingham
'Monoparin' Anticoagulant Heparin	QE Hospital Pharmacy, Birmingham, England
Normal human serum (AB+)	Sigma, Poole, England

Partially purified protein	Statens Seruminstitut, Copenhagen, Denmark
Penicillin	QE Hospital Pharmacy, Birmingham, England
Phytohaemagglutinin	Murex Diagnostics, Dartford, England
Printed filter mats	Pharmacia Biotechnology Ltd, Milton Keynes, UK
Rabies protein	A kind gift from IDIS, Germany
RPMI 1640	GibcoBRL, Life Technologies Ltd, Scotland
'Sigmacoat' silicon	Sigma, Poole, England
Streptomycin	Evans Medical, Horsham, England
Tetanus Toxoid	Statens Seruminstitut, Copenhagen, Denmark
^{3}H Thymidine (1mCi/ml)	Amersham International, Aylesbury, England
Trypan Blue solution	Sigma, Poole, England

2.3 Antibodies for purification and cell staining

CD molecule*	Clone	Isotype	Conjugate	Supplier
CD8	RFT8	IgG1	unlabelled	Royal Free Hospital, London
CD11b	OKM1	IgG1	unlabelled	Ancell, Bayport, USA
CD14	MON1040		unlabelled	Bradshaw Biologicals
CD16	0813		unlabelled	Immunotech, Fullerton, USA
CD19	RFTB9	IgG1	unlabelled	Royal Free Hospital, London
CD45RA	SN130		unlabelled	Royal Free Hospital, London
CD45RO	UCHL1		unlabelled	Royal Free Hospital, London
Glycophorin A				Pharmingen, San Diego, USA
CD3	UCHT1	IgG1	PE	Dako Ltd, Bucks, England.
CD4	Leu3a	IgG1	FITC	Becton Dickinson, Oxnard, USA
CD4	Leu3a	IgG1	PE	Becton Dickinson, Oxnard, USA
CD8	BH7	IgG1	FITC	Serotec, Kidlington, Oxford
CD14	TUKd	IgG2a	PE	Caltag, Burlingame, California
CD16	DJ180	IgG1	FITC	Dako Ltd., Bucks, England
CD19	HD37	IgG1	PE	Dako Ltd., Bucks, England

CD45RO	UCHL1	IgG2a	PE	Dako Ltd., Bucks, England
CD45RA	2H4	IgG1	PE	Coulter, Luton, England
Irrelevant		IgG1	FITC	Dako Ltd., Bucks, England
Irrelevant		IgG1	PE	Dako Ltd., Bucks, England.
Irrelevant		IgG2a	PE	Dako Ltd., Bucks, England

* CD molecules are mouse anti - human monoclonal antibodies.

2.4 Enzyme linked immunosorbant assay

Anti - β_2GP1 IgG ELISA kit	Shield Diagnostics, Dundee, Scotland
Anti - β_2GP1 ELISA Kit	Genesis Diagnosis, Cambridge, UK
Anticardiolipin ELISA kits	Binding Site, Birmingham, England
Anti - La Ig ELISA kits	Shield Diagnostics, Dundee, Scotland
Mouse anti - human IgG(Fc)	Binding Site, Birmingham, England
Nunc maxisorp ELISA plates	Nunc, Denmark
Tween 20	BDH Laboratories, Poole, England
TT coated ELISA plates	Binding Site, Birmingham, England

2.5 Extraction of La protein

Antipain	Sigma, Poole, England
Chymostatin	Sigma, Poole, England
Dithiothreitol	Sigma, Poole, England
EDTA	Fisons, Loughborough, England
Glycerol	BDH Laboratories, Poole, England
Leupeptin	Sigma, Poole, England
Nonidet P40	BDH Laboratories, Poole, England
Pepstatin A	Sigma, Poole, England
PMSF	BDH Laboratories, Poole, England

2.6 PAGE and Electrophoretic transfer to nitrocellulose membrane

Acrylamide : Bisacrylamide	Biometra Ltd., USA
Ammonium persulphate	BDH Laboratories, Poole, England
Avidin complex	Dako Ltd., Bucks, England
BCIP	Sigma, Poole, England
Bovine serum albumin	Sigma, Poole, England
Bromophenol Blue	BDH Laboratories, Poole, England
Coomassie Blue G	Sigma, Poole, England
Glacial acetic acid	Fisons, Loughborough, England
Glycine	BDH Laboratories, Poole, England
Methanol	Fisons, Loughborough, England
Mouse anti - La mAb, SW5	A kind gift from Dr. Pruijn
Nitroblue tetrazolium	Sigma, Poole, England
PVDF Trans-Blot transfer membrane	Biorad, Hercules, California, USA
Rabbit anti - mouse antibody	Dako Ltd., Bucks, England
Recombinant human La	A kind gift from Dr. Pruijn
'Seablue' molecular weight standard	Novel Experimental Technology, San Diego, USA.
Sodium dodecyl sulphate	GibcoBRL, Life Technologies Ltd., Scotland
Swine anti - rabbit biotin conjugated	Dako Ltd., Bucks, England
TEMED	Sigma, Poole, England
Tris base	GibcoBRL, Life Technologies Ltd., Scotland
Vivaspin concentrator	Vivascience, Lincoln, UK

Chapter 3

Methods

3.1 Subjects

Patients were selected from the cohort of 200 who attended the SLE clinic at the Queen Elizabeth Hospital, Birmingham and who fulfilled the ARC criteria (1982) for the classification of SLE (Tan *et al.*, 1982). The majority of patients were taking some medication which included either hydroxy - chloroquine, prednisolone, azathiopine or methotrexate (see Appendix A). Patients were selected on the basis of serum antibodies (see section 3.2) and T cell proliferative responses to recall antigens (see section 3.3.9).

Healthy volunteers were either members of the Division of Immunity and Infection or were blood donors to the Tissue Typing Laboratories of the West Midlands Blood Transfusion Service. These control subjects were not on any medication at the time of the study.

HLA DR and HLA DQ genotyping was performed by PCR (Oxford Tissue Typing Unit, Oxford and Department of Medicine, University of Birmingham).

Approval for this study was obtained from the local ethics committee. All of the subjects enrolled in the study were Caucasian.

3.2 Enzyme linked immunosorbant assay

The presence of anti - La and anti - Ro antibodies was determined using countercurrent immunoelectrophoresis as part of the patients clinical diagnosis. Anti - La antibody titres were confirmed using a commercial ELISA kit which has a cut off point of 2. Lupus anticoagulant was determined as part of the patients clinical diagnosis. Clinical diagnosis tests were carried out by the Clinical Immunology Service, University of Birmingham.

3.2.1 Total IgG ELISA

Nunc maxisorp 96 well ELISA plates were coated with 50μl mouse anti - human IgG (Fc) diluted 1/100 in carbonate buffer, pH 9.6 (1.59g sodium carbonate and 2.94g sodium hydrogen carbonate in 500ml distilled water). Plates were incubated overnight at 4°C. The wells were washed three times with PBS (6g disodium hydrogen orthophosphate, 0.5g sodium dihydrogen orthophosphate and 8.8g sodium chloride in one litre of distilled water). Wells were washed using an automated plate washer. The wells were then blocked for one hour at 37°C with blocking buffer (3% BSA and 0.05% Tween 20 in PBS). After washing three times with wash buffer (0.05% Tween 20 in PBS) the wells were incubated with either a standard control sample obtained from Birmingham Clinical Immunology Service or patient serum. Patient serum was diluted 1/50 in diluent (1%BSA and 0.05% Tween 20 in PBS). All samples were tested in duplicate. Plates were incubated for one hour at 37°C before washing three times with wash buffer. Sheep anti - human IgG conjugated with horse - raddish peroxidase (HRP) was diluted 1/1000 in diluent and added to all of the wells (50μl per well). Plates were incubated for a further hour at 37°C and washed again three times in wash buffer. Plates were developed with ODP (50μl per well). The reaction was stopped after ten minutes with 1M sulphuric acid (55.6ml 18M sulphuric acid in 1 litre distilled water) and the optical density measured at 492nm using a model 450 microtitre plate reader. A standard curve was plotted of the control serum and this was used to calculate the concentration of antibody in the patient sera. The control serum obtained from a healthy adult contained 13.17mg/ml IgG and 1.35mg/ml IgM.

3.2.2 Anti - Tetanus toxoid antibody ELISA

Plates pre - coated with tetanus toxoid were obtained from The Binding Site, Birmingham. A standard anti - tetanus toxoid control sample was obtained from the Scottish Blood Transfusion Centre. The standard control sample or patient serum (diluted 1/50 in diluent) was added to the plate in 50μl. The ELISA was then carried out as described for total IgG (see section 3.2.1). The standard control sample was tested between 100μg/ml and 1μg/ml. A standard curve was plotted of the control serum and this was used to calculate antibody

concentrations in the patient sera.

3.2.3 Anti - β2 Glycoprotein 1 ELISA

Serum β2GP1 antibody titres were determined using two commercial ELISA kits (Genesis Diagnostics and Shield Diagnostics) and carried out according to the manufacturer's instructions. Serum was considered positive in the Genesis and Shield kits if the titre was ≥ 5 Units/ml and ≥ 10 Units/ml, respectively. Antibody levels were tested at two different time points to examine any change in antibody titre with time. Serum was collected on the day of the T cell proliferation assay or taken during the previous 6 months to 3 years and stored at -80°C. The frozen aliquots were obtained from Dr. C. Gordon who collected the serum for clinical studies. Sera were also tested at Imperial College, London (Dr. Kevin Davies) and the Methodist Hospital of Indiana, Indianapolis (Dr. Dawn Wagenknecht).

3.2.4 Anticardiolipin antibodies

Sera were tested for anticardiolipin antibodies using a commercial ELISA kit (The Binding Site, Birmingham). The test was carried out according to the manufacturer's instructions. Serum was considered positive if the antibody titre was ≥ 11 Units/ml.

3.3 Tissue Culture

3.3.1 Culture conditions and media

All cell manipulations were performed in a tissue culture class II safety cabinet. Human cell lines and PBMC's were cultured in RPMI 1640 supplemented with 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin. This medium will be referred to as complete medium. Foetal calf serum was added to the complete medium for culturing cell lines. Normal human AB serum was added to the complete medium for culturing PBMC's. Serum was heat inactivated at 56°C for 45 minutes before use.

The SW5 hybridoma (a kind gift from Dr. Pruijn, University of Nijmegen, Netherlands) was grown in DMEM supplemented with 1% glutamine, 1mM sodium pyruvate, 100U/ml penicillin and 100μg/ml streptomycin. The supernatant was used as a source of anti - La monoclonal antibody. Cells were maintained between 0.2x10^6/ml and 1x10^6/ml in a humidified gassed (5% $C0_2$, 95% air) environment at 37°C.

3.3.2 Cell counts

To check the viability of cells equal volumes (10μl) of cell suspension and trypan blue stain were mixed together. The cells were loaded on to a haemocytometer (Weber Scientific International) and viewed under a light microscope (Olympus, Tokyo, Japan). Blue cells, which are unable to exclude trypan blue, were scored as non - viable. The percentage of living cells which do not take up trypan blue was determined by counting at least 100 cells. The number of cells per ml was calculated using the following equation:

$$\text{No. viable cells/ml} = \frac{\text{Number of viable cells in 25 squares x Trypan blue dilution factor}}{1\text{x}10^{-4}}$$

3.3.3 Cryopreservation of cells

Cells were centrifuged at 500xg for 8 minutes and resuspended in cold RPMI 1640 at 10 - 40x10^6/ml. An equal volume of cold freezing mix (15% dimethyl sulphoxide and 85% heat inactivated FCS) was added drop wise with gentle agitation. Cells were frozen in 1ml cryovials. The aliquots were placed in the header of the liquid nitrogen tank for at least 3 hours and then transferred to storage canes in the liquid nitrogen phase.

3.3.4 Thawing cells from liquid nitrogen

Cells were thawed rapidly by placing the cryovial in a 37°C water bath and gently shaking the tube. Cells were transferred to sterile plastic 25ml universals and 20ml of warm RPMI 1640 was added drop wise with periodic mixing. The cells were washed by centrifuging at

500xg for 8 minutes. The pellet was resuspended in RPMI 1640. The washing procedure was repeated three times and the viability checked with trypan blue exclusion (see section 3.3.2).

3.3.5 Separation of peripheral blood mononuclear cells

Mononuclear cells were obtained by Ficoll - Paque density centrifugation from heparinised fresh blood. Blood was diluted 1:1 with sterile PBS, layered on to 8ml of Ficoll - Paque in a sterile 25ml plastic universal and centrifuged at 500xg for 30 min. Mononuclear cells were collected from the interface and washed three times in RPMI 1640 at 500xg for 10 minutes. The final pellet was resuspended in complete medium supplemented with 10% normal human serum. Cells were used fresh in proliferation assays. Viability was checked using trypan blue exclusion (see section 3.3.2).

3.3.6 T cell purification

Mononuclear cells were obtained by Ficoll - Paque density centrifugation (see section 3.3.5). Adherent cells were removed from the mononuclear cells and used as antigen presenting cells (see section 3.3.7). Two rounds of adherence was performed. The non - adherent cells were collected into human serum coated round bottom tubes. The tubes were coated with human serum at 37°C for 30 minutes. Petri dishes were washed over three times with RPMI 1640 to obtain the maximum number of non - adherent cells.

CD4+ T cells were negatively selected from the non - adherent cells using a cocktail of antibodies and magnetic beads (Dynalbeads). All steps were carried out on ice to prevent internalisation of the antibody. Additionally, T cells were collected into human serum coated tubes to prevent them from adhering to the plastic. The non - adherent cells were centrifuged at 500xg for 8 minutes and the pellet was incubated with an antibody cocktail for 30 minutes on ice. The antibody cocktail was prepared in 500μl RPMI 1640 and contained saturating amounts of unlabelled anti - CD19, CD14, CD16, CD11b, CD8 antibody and glycophorin A. Antibody concentrations were based upon the ability of the

antibody to bind PBMC's, as analysed by flow cytometry. The antibody cocktail was filtered through a 0.2μm acrodisc filter before adding to the cells. After labelling the cells with the antibody cocktail the cells were washed twice in cold RPMI to remove excess antibody. The cells were then resuspended gently in 100μl of RPMI. One round of depletion with goat anti - mouse IgG coated Dynalbeads was performed using 100μl of beads in 1ml of RPMI. The Dynalbeads were washed in RPMI before use to remove unbound goat anti - mouse antibody and sodium azide. The cells were incubated with the Dynalbeads for 30 minutes on ice and mixed gently every 10 minutes. During the incubation the Dynalbeads bound to the antibody which was bound to the cells. RPMI (5ml) was added to the cells and the tube was then lined up against a magnet for one minute. The Dynalbeads were attracted to the side of the tube which was aligned with the magnet. The medium containing the CD4 T cells was aspirated using a 5ml sterile pipette and collected into human serum coated tubes. In order to obtain a maximum yield of cells the Dynalbeads were washed twice with 10ml RPMI using the magnet to separate the Dynalbeads from the unlabelled CD4 T cells. The CD4 T cells were pelleted and resuspended in RPMI.

Purified CD4 T cells were used to prepare purified CD45RA T cells. CD4 T cells were incubated with saturating concentrations of anti - CD45RO antibody (UCHL1) in 500μl RPMI. The cells were mixed gently every 10 minutes for 30 minutes and then washed three times with cold RPMI at 500xg for 8 minutes. The antibody labelled cells were incubated with Dynalbeads and then the tube was lined up against a magnet as described above for the purification of CD4 T cells. The Dynalbeads were washed three times in order to obtain the maximum yield of unlabelled cells. The medium containing the unlabelled CD45RA T cells was collected into human serum coated tubes. The CD45RA T cells were pelleted at 500xg for 8 minutes and incubated with 50μl Dynalbeads in 1ml RPMI. After five minutes the cells were centrifuged at 100xg for 3 minutes. After a further five minutes the Dynalbeads were gently resuspended. This process of spinning down the cells and resuspending was repeated three times. RPMI (5ml) was added to the cells and the tube was lined against a magnet for one minute. The medium containing the CD45RA T cells was collected into human serum coated tubes. The beads were washed three times

to obtain the maximum yield of cells. The CD45RA T cells were pelleted at 500xg for 8 minutes and resuspended in 5ml RPMI. To remove residual Dynalbeads the tube was lined up with the magnet as described above. CD45RA T cells were collected then centrifuged and the pellet resuspended in complete medium containing 10% human serum. Three rounds of depletion was necessary to obtain purified CD45RA T cells from PBMC's. The purity of the cells was determined by flow cytometry (see section 3.3.8). The cell viability was checked by trypan blue exclusion (see section 3.3.2).

CD45RO T cells were prepared as described for the purification of CD45RA T cells except that anti - CD45RA antibody (SN130) was added to the CD4 T cells in place of the anti - CD45RO antibody.

3.3.7 Preparation of antigen presenting cells

Mononuclear cells were obtained by Ficoll - Paque density centrifugation (see section 3.3.5). The PBMC's were incubated on a sterile human serum coated petri dish for 30 minutes at 37°C. The petri dish was pre - coated with human serum for 30 minutes at 37°C. Non - adherent cells were collected into human serum coated tubes and used as a source of T cells. Adherent cells were scraped off the petri dish with the rubber tip of a 5ml sterile syringe plunger and resuspended in RPMI. This was repeated twice in order to obtain the maximum yield of adherent cells. Adherent cells were collected into siliconised tubes to prevent them from adhering to the plastic. Tubes were pre - coated with sterile 'Sigmacoat' for 2 minutes and washed with sterile PBS before use. The petri dish was examined on an inverted microscope to ensure that all the adherent cells had been removed. The adherent cells were washed in RPMI and resuspended in 500μl complete medium supplemented with 10% human serum. The adherent cells were irradiated at 3500 rads (Physics department, University of Birmingham) to prevent them from proliferating in response to antigenic stimulation and then washed three times with RPMI to remove oxygen free radicals. Cells were finally resuspended in complete medium supplemented with 10% human serum.

3.3.8 Flow cytometry

Cell purity was determined by flow cytometry. CD45RA or CD45RO T cells were divided into FACS tubes ($2x10^5$ cells per tube) and centrifuged at 500xg for 8 minutes at 4°C. The medium was aspirated and the cells were resuspended by gently flicking the tube. Optimal concentrations of the relevant antibody were added to the cells and then incubated on ice for 30 minutes. The antibody was diluted in PBS containing 1% BSA (PBS/BSA) and added to the cells in 100μl. The cells were stained with irrelevant antibody, anti - CD3, CD4, CD8, CD14, CD16, CD19, CD45RA or CD45RO antibody either as a single or double stain. Antibody concentrations were based upon the optimum binding to PBMC's. The cells were washed twice in cold PBS/BSA, resuspended in 500μl PBS/BSA and analysed on a Coulter FACStar flow cytometer.

3.3.9 PBMC proliferation assay

Freshly isolated cells (see section 3.3.5) were cultured in flat bottom 96 well plates at $2x10^5$ cells per well together with the optimal concentration of antigen (see section 3.3.11) and anti - CD28 monoclonal antibody. Anti - CD28 antibody was used at a final concentration of 0.05μg/ml. Tests were set up in triplicates for recall antigens and mitogens, and in a minimum of five replicates for the other antigens. Cultures were incubated for 7 days at 37°C in 5% CO_2 humidified atmosphere. Wells were pulsed for the final 18hr of culture with 0.4μCi tritiated thymidine per well and harvested on to printed filters mats using a cell harvester (Scatron, Newmarket, England). The filter mats were placed into a plastic envelope and 10ml of scintillant fluid was added. The bag was sealed and the thymidine incorporation was measured using a β - scintillation counter (1206 multigamma II counter, Wallac). Results were presented as counts per minute (CPM).

3.3.10 T cell proliferation assay

$5x10^4$ T cells and $5x10^3$ adherent cells were co - cultured in flat bottom 96 well microtitre plates with or without antigen plus anti - CD28 monoclonal antibody at a final

concentration of 0.05μg/ml. Wells were set up in triplicate. Cells were incubated for 7 days at 37°C in 5% CO_2 atmosphere. For the final 18 hours wells were pulsed with 0.4μCi tritiated thymidine per well and harvested as described in section 3.3.9.

3.3.11 Antigens and mitogens

PHA was used at a final concentration of 1μg/ml and rabies protein at 5μg/ml. All remaining antigens including PPD, tetanus toxoid, β_2 glycoprotein 1, synthetic peptides and partially purified human native La were used at a final concentration of 10μg/ml. Rabies protein, partially purified La and β_2 glycoprotein 1 were dialysed against PBS overnight and passed through a 0.2μm acrodisc filter before using in the cell assays. Rabies protein was a gift from IDIS and prepared from cell cultures of a human diploid cell line. β_2 glycoprotein 1 was a kind gift from Serbio and was prepared from human plasma by barium citrate adsorption, ion exchange and heparin chromatography. Additionally, β_2 glycoprotein 1 was kindly donated by IDRL and was prepared from human plasma by ethodin, sodium chloride and perchloric acid precipitation steps and purified by affinity chromatography on a heparin column. Synthetic peptides were made by Alta Bioscience or Genosys Biotechnologies using automated multiple peptide synthesis and FMOC chemistry. The peptides were solubilised in 1ml PBS or 100μl DMSO plus 900μl PBS for those peptides which did not dissolve in PBS alone.

3.3.12 Protein determination

The bicinchonic acid (BCA) assay was used to determine the concentration of proteins and peptides. The assay was carried out according to the manufacturers instructions. Protein and peptide concentrations were determined by comparison to a standard curve using bovine serum albumin titrated from 2mg/ml.

3.3.13 Statistical analysis

A proliferative response was considered to be positive if the stimulation index (SI) was

greater than or equal to three. A stimulation index was calculated as follows:

$$\text{Stimulation index (SI)} = \frac{\text{CPM of antigen stimulated well}}{\text{CPM of average unstimulated wells}}$$

Significance levels were determined using a two tailed unpaired student's T Test. A value of $p \leq 0.05$ was considered significant.

Where appropriate phenotype frequencies of HLA alleles were compared between healthy individuals and patients using a Fisher's two tailed exact test. P values are reported uncorrected and corrected for the number of HLA alleles at a particular locus. For HLA DR uncorrected *P* values were multiplied by 10, for HLA DQ values were multiplied by 5 and for HLA DQβ1* alleles values were multiplied by 14.

3.4 Preparation of La antigen

3.4.1 Extraction of La from human cell lines

Dignam's Buffer A — 100mM Hepes, pH 7.9; 1.5mM $MgCl_2$; 10mM KCl; 0.5mM DTT; 0.05% nonidet P-40

Dignam's Buffer C — 5mM Hepes, pH 7.9; 26% glycerol; 1.5mM $MgCl_2$; 0.2mM EDTA; 0.5mM DTT; 420mM NaCl

Cell lines were maintained at 37°C in complete medium supplemented with 10% heat inactivated FCS. For bulk culture, cells were grown in spinner flasks in complete medium supplemented with 10% heat inactivated FCS and 1% HEPES buffer. Cells were harvested by centrifugation at 500xg for 8 minutes and washed in cold PBS. Cells were counted using trypan blue exclusion (see section 3.3.2). Protein was extracted from the cell lines according to Dignam's methodology (Dignam *et al.*, 1983). Cells were centrifuged at

500xg for 8 minutes. For every $1x10^7$ cells 100μl of buffer A containing 0.5μg/ml of each of the proteases; chymostatin, leupeptin, antipain, pepstatin A and 1mM PMSF was added to the cell pellet. The cells were centrifuged at 13000rpm for 10 seconds using a Micro Centaur eppendorf centrifuge. The supernatant was removed and an equal volume of buffer C was added. This preparation was stored at -20°C and referred to as the cytoplasmic portion. To the pellet 100μl buffer C containing 0.5μg/ml of each of the proteases; chymostatin, leupeptin, antipain, pepstatin A and 1mM PMSF was added and the sample left on ice for 15 minutes. The sample was then centrifuged at 13000rpm, 4°C for 5 minutes. The supernatant was collected and stored at -20°C. This supernatant contained extractable nuclear proteins while the residual pellet contained DNA. The cytoplasmic portion and extractable nuclear proteins were used as a source of La protein. The presence of La protein was demonstrated using SDS Page electrophoresis and Western blots (see section 3.4.3 - 3.4.5).

3.4.2 Purification of La from cell line K562

Buffer A	3mM $MgCl_2$; 0.1mM EDTA; 0.5mM DTT; 25mM Tris. Adjust pH of cold buffer with HCl to pH8
Buffer B	Buffer A containing 100mM NaCl
Buffer C	Buffer A containing 500mM NaCl
Buffer D	Buffer A containing 1M NaCl

La was purified from the K562 cell line using ion exchange chromatography on a DEAE Sepharose Fast Flow matrix followed by a heparin affinity column (Pharmacia). The purification was performed at 4°C to minimise protease activity. The method used was based upon Stefano (1984). Protein extract from the K562 cell line (see section 3.4.1) was placed in dialysis tubing and treated with aquacide overnight to reduce the volume. The cell extract was then dialysed overnight into buffer A. The extract was passed through a 0.2μm acrodisc filter before loading on to the ion exchange chromatography column.

The DEAE column was cleaned with five column volumes of buffer D and equilibrated

with ten column volumes of buffer A. The column was run at 4ml/min. Once the baseline was established the K562 cell extract was loaded in buffer A. Unbound proteins were washed off with buffer A - the 'flow through' fraction. Bound proteins were eluted with buffer B and buffer C. Buffer C was collected since it contained the La protein. The column was washed with buffer D to remove residual proteins and stored in 20% ethanol.

The fraction eluted with buffer C was dialysed immediately into buffer A to minimise the disruption to the protein structure by the salt content. This fraction was then loaded onto a pre - packed heparin column in buffer A. The heparin column was cleaned with buffer D and equilibrated with buffer A before use. The column was run at 1ml/min. Bound protein was eluted with buffer D. The flow through fraction and the fraction containing bound protein was collected and dialysed overnight into PBS since both fractions contained La protein. The presence of La protein was demonstrated using SDS Page electrophoresis and Western blots (see section 3.4.3 - 3.4.5). Both fractions however contained additional proteins. According to Stefano (1984) the heparin flow through fraction contained RNA - bound La and the buffer D fraction contained RNA - free La protein. RNA - free La was used in the proliferation assays after it had been concentrated using a vivaspin concentrator with a 30kDa molecular weight cut off, dialysed overnight into PBS and filter sterilised.

3.4.3 SDS - PAGE electrophoresis

Gel constituents	10% gel	3% Stacker gel
Acrylamide : Bisacrylamide	2.5ml	1.45ml
1.5M Tris, pH8.8	2.5ml	0ml
0.5M Tris, pH6.8	0ml	2.5ml
Distilled water	4.9ml	11.8ml
10% SDS	0.1ml	0.1ml
TEMED	3µl	10µl
10% Ammonium persulphate	30µl	50µl

1.5M Tris, pH8.8 — 36.3g Tris in 200ml distilled water adjusted to pH 8.8 with concentrated hydrochloric acid

0.5M Tris, pH6.8	12.1g Tris in 200ml distilled water adjusted to pH 6.8 with concentrated hydrochloric acid
Non reducing Sample buffer	4ml 10% SDS; 2.5ml 0.5M Tris, pH6.8; 2ml glycerol; 1.5ml distilled water; add a few grains of bromophenol blue
SDS - Page Running buffer (x5)	30g Tris; 144g Glycine; 10g Sodium dodecyl sulphate made to 2 litres with distilled water
Gel fixative	10ml acetic acid; 50ml distilled water; 40ml methanol
Coomassie Blue Stain	50mg Coomassie Blue G; 20ml acetic acid made up to 200ml with distilled water
Coomassie Blue destain	10ml acetic acid in 100ml distilled water

A 5cm 10% polyacrylamide running gel (acrylamide : bisacrylamide 37.5:1) with a 1cm 3% stacking gel was prepared as described in accordance with the method of Laemmli (1970). Saturated butanol was layered above the running gel while it was setting to ensure an even surface and to prevent air reaching the gel. A plastic comb was inserted into the stacker gel to form the wells. The gel was assembled in the electrophoresis tank and running buffer added. Protein was loaded into the wells in 20μl. Protein samples were prepared in non reducing sample buffer. The gel was run at 150V (and 80mA) for 45 minutes or until the leading edge of the dye reached the bottom of the gel. Proteins were either transferred onto nitrocellulose membrane (see section 3.4.4) or fixed for 30 minutes with gel fixative, stained with Coomassie Blue for 2 hours and destained overnight. Coomassie stained gels were dried for approximately 2 hours using a gel drying apparatus.

The molecular weights of the protein samples were estimated from the migration of 'See-Blue' pre-stained protein standards having molecular weights (kDa) 250 (myosin), 98 (BSA), 64 (glutamic dehydrogenase), 50 (alcohol dehydrogenase), 36 (carbonic

anhydrase), 30 (myoglobin), 16 (lysozyme), 6 (aprotinin) and 4 (insulin β chain).

3.4.4 Western blotting

Transfer buffer	2.93g glycine; 5.81g Tris; 0.375g SDS; 200ml methanol made to 1 litre with distilled water

Proteins were transferred on to PVDF Trans - Blot transfer membrane at 0.8mA/cm^2 for 2 hours using the semi-dry LKB 2005 transfer electroblotting unit (Towbin *et al.*, 1979). Six sheets of Whatman filter paper cut to the same size as the gel were soaked in transfer buffer and layered on to the anode of the electroblotting unit. PVDF membrane was presoaked in methanol for 10 seconds, to improve its capacity to bind proteins, washed in transfer buffer and placed on top of the filter paper. The gel was then added to the stack followed by six further sheets of filter paper. Air bubbles were pushed out of the stack by rolling a pencil over the surface. The cathode was placed on to the electroblotting unit and the current was applied. After 2 hours the membrane was removed and either stored in 'Saranwrap' at 4°C or probed with specific antibodies (see section 3.4.5).

3.4.5 Detection of La protein bound to nitrocellulose

BCIP/NBT reagent	33μl NBT (50mg/ml in dimethylformamide); 17μl BCIP (in DMSO) dissolved in 5ml 100mM Tris, pH 9.5; 100mM NaCl; 5mM $MgCl_2$

The PVDF membrane was blocked with PBS containing 3% BSA (PBS/3%BSA) overnight at 4°C to prevent non - specific binding of antibodies. The membrane was washed three times, each for 5 minutes, with PBS then incubated with SW5 cell supernatant (containing anti - La antibody) for 1 hour. After washing with wash buffer (PBS containing 0.1% Tween 20) three times the membrane was incubated with rabbit anti - mouse antibody for 1 hour. The membrane was washed and incubated with biotinylated conjugated swine anti - rabbit antibody. All the antibodies were diluted 1/1000 in PBS

containing 0.1% Tween 20 and 1% BSA. After washing the blot was incubated with avidin complex kit prepared in 0.05M Tris buffer, pH 7.9, according to the manufacturers instructions. After 45 minutes the membrane was washed three times. Membranes were developed with the BCIP/NBT reagent and washed in distilled water once the purple colour had developed. The membrane was air dried then stored in the dark.

Chapter 4

Results Section 1

Proliferative Response to Recall and Naive Antigens in Patients with SLE

Many investigators have previously demonstrated an altered T cell proliferative response to PHA, anti - CD2 antibody and anti - CD3 antibody in patients with SLE compared to healthy individuals (see section 1.4.5). Very little work has been reported using biologically relevant antigens. In the few reported studies patients with SLE show a poor proliferative response to the antigens tested. The aim of this section of work was therefore to establish an immunological assay for measuring optimal proliferative responses in patients with SLE and healthy individuals. Two recall antigens, tetanus toxoid and partially purified protein derivative of tuberculin, and a non recall antigen, rabies protein were studied to determine if a primed and a naive response could be differentiated in patients with SLE.

Adding anti - CD28 antibody to the cell cultures, removing the macrophage population or resting the cells prior to antigenic stimulation have been reported to improve the proliferative response in patients with SLE (see section 1.4.5). The second aim of this section of work was therefore to examine these published reports and to determine if the proliferative response in patients with SLE can be restored. Any changes measured in the proliferative response by these methods may suggest a mechanism for the cell defects reported in SLE.

Proliferative responses to recall antigens are reported to be lower in patients with SLE compared with healthy individuals (see section 1.4.5). However, the studies do not state if the subjects had been previously exposed to the antigen being studied. Previous exposure affects the magnitude of response - a primary response (no previous exposure) is generally weaker and the kinetics are slower compared to a secondary (or recall) response. It was therefore necessary to compare the proliferative response to the recall antigens with vaccination status in order to interpret the presence and absence of a response.

4.1 Development of a proliferation assay

Initial experiments were designed to determine optimal assay conditions with respect to antigen concentration and proliferation kinetics. The assay was developed using standard antigens.

4.1.1 Determination of the optimal antigen concentrations

An essential part in the development of an assay system for measuring cell proliferation is the determination of optimal antigen concentrations. Antigens used as standards in this study were tetanus toxoid and PPD as recall antigens, rabies protein as a naive T cell antigen and PHA as a mitogen. The concentration of PHA had previously been determined within the department as 1μg/ml and was used to confirm that T cells were responding to stimuli. For the remaining antigens, PBMC's taken from patients and healthy individuals were cultured with a range of concentrations for each antigen (Figure 4.1). The maximum proliferation using tetanus toxoid and PPD was found to be 10μg/ml, while for rabies protein maximum proliferation occurred using 5μg/ml. The experiments were confirmed using five healthy individuals and four patients with SLE. The antigen concentrations determined in these experiments were used in the remainder of this study.

4.1.2 Kinetics of cell proliferation

The kinetics of the assay was examined to confirm that optimum proliferation responses were measured. Assays were established using the optimal concentrations of antigens and $2x10^5$ cells per well. Triplicate wells were set up and cultured for either 5, 7 or 9 days. Proliferation was measured by thymidine uptake during the final 18 hours of culture. As shown in Figure 4.2, the peak proliferative response to tetanus toxoid, PPD and rabies protein occurred on day 7 for the three healthy individuals and three patients studied. The proliferative response to the antigens was always lower in the patients but all of the subjects responded to PHA (see Appendix A). On the basis of these findings a 7 day

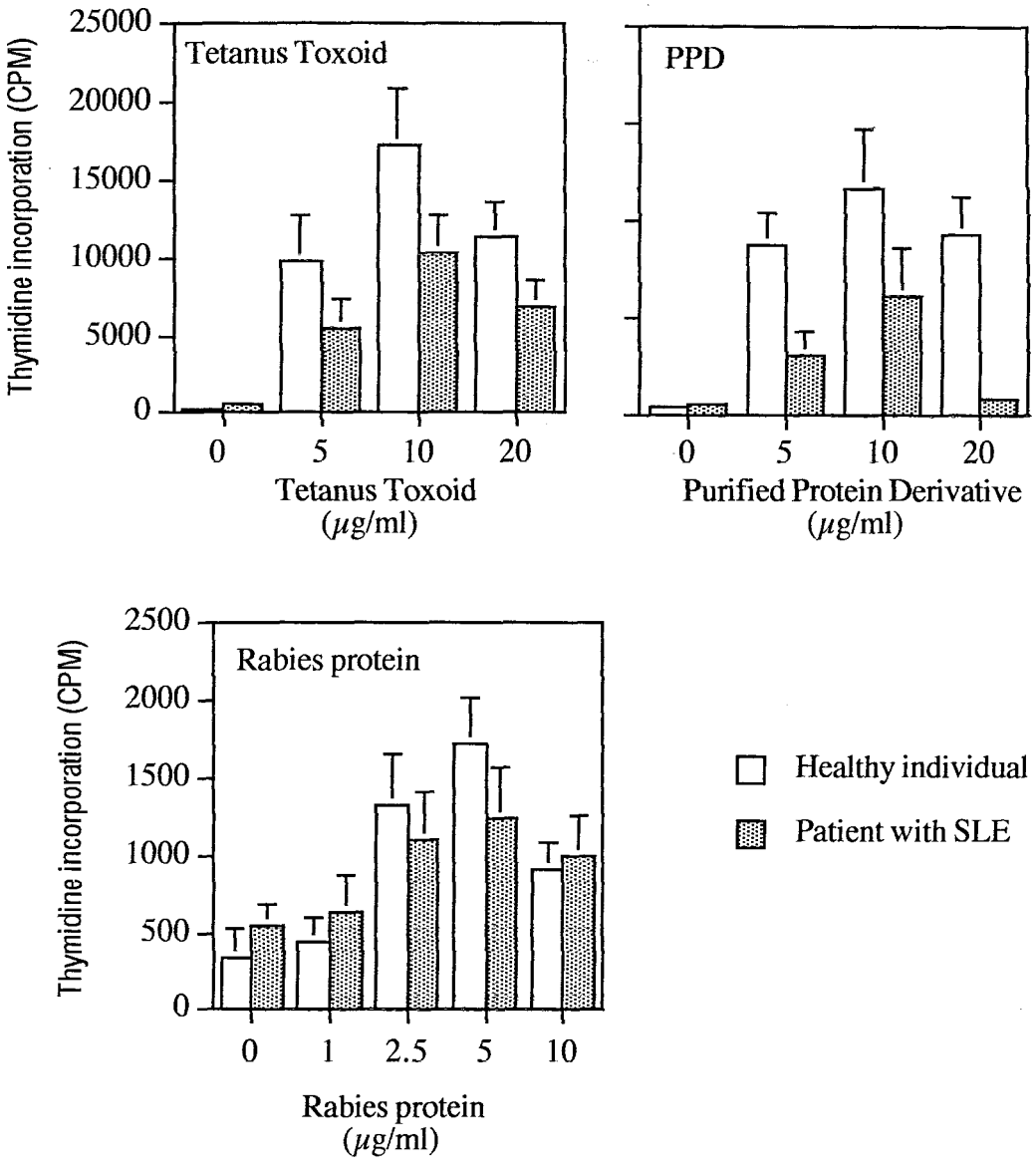


Figure 4.1

Proliferative responses (tritiated thymidine incorporation) obtained from five healthy individuals and four patients with SLE. Triplicate cultures of $2x10^5$ cells were incubated for 7 days with either tetanus toxoid, purified protein derivative or rabies protein (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

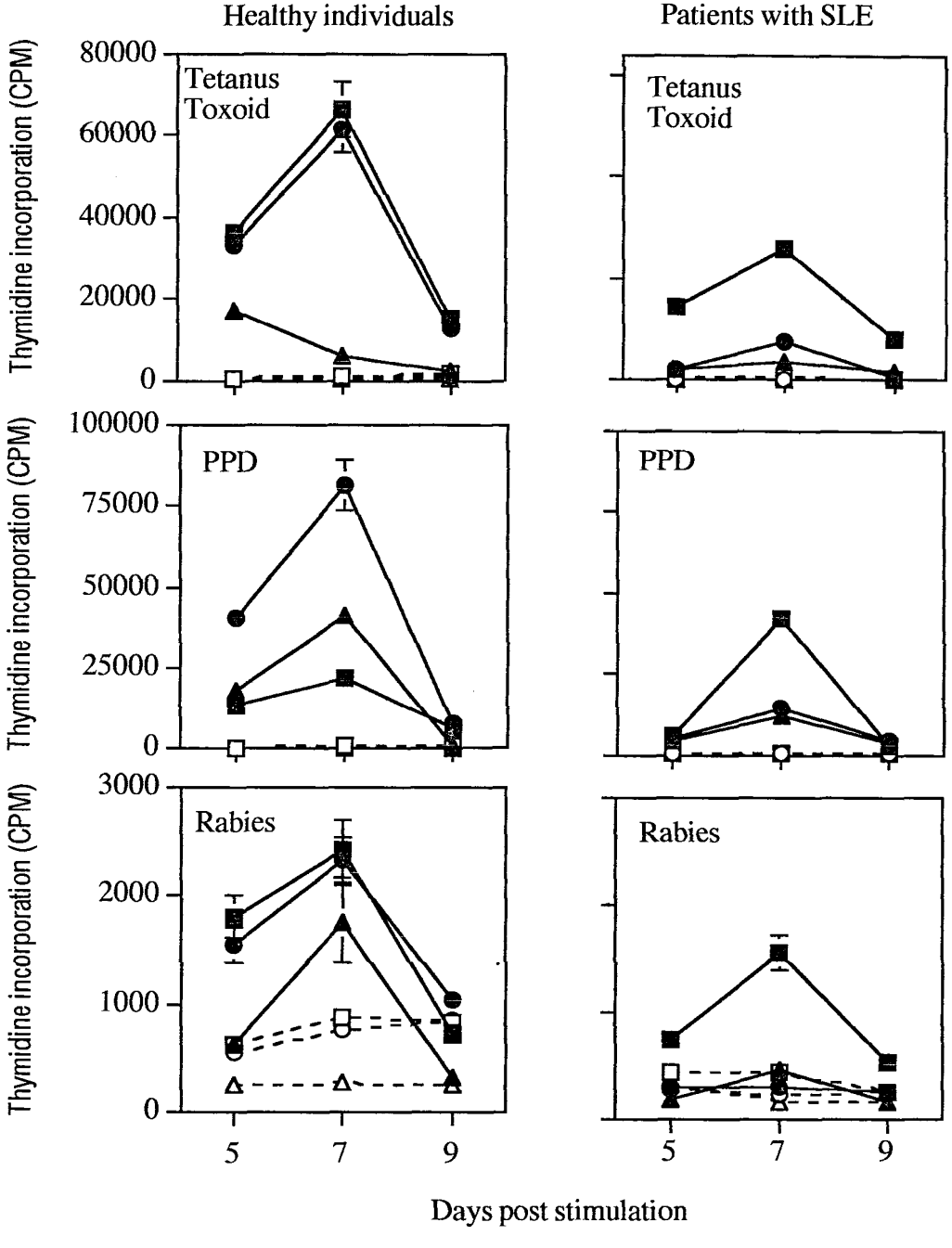


Figure 4.2

PBMC's from three healthy individuals and three SLE patients were cultured with either tetanus toxoid, PPD or rabies protein for 5, 7 or 9 days (see section 3.3.9). Black symbols represent responses to antigen. Clear symbols represent responses to medium only. Thymidine incorporation was measured during the final 18hr of culture. The results are expressed for each individual as mean CPM of the triplicates ± standard deviation.

culture was adopted for all PBMC assays used in this study.

4.1.3 The effect of cell concentration

It was noted that fewer PBMC's were obtained from the same volume of blood taken from patients compared to healthy individuals. There was also a reduced *in vitro* proliferative response to antigen in patients compared to healthy individuals. It was therefore essential to determine the optimum cell concentration required to obtain measurable cell proliferation in the patients. Cultures were established using fresh PBMC's taken from three healthy individuals and three patients at either $1x10^5$, $2x10^5$ or $4x10^5$ cells per well. The peak response to tetanus toxoid and PPD was seen using $2x10^5$ cells per well (Figure 4.3).

4.1.4 The effect of human serum and foetal calf serum

Proliferation is normally supported by serum added to the assay (Rumley *et al.*, 1984). Human serum and foetal calf serum (FCS) were screened to identify which one ensured a low background response whilst still supporting a strong antigen driven response. Cultures were established in RPMI supplemented with either 10% human serum or 10% FCS. Serum was heat inactivated before use (see section 3.3.1). The proliferative response, without the addition of antigen, was higher in wells containing PBMC's plus FCS compared to human serum (Figure 4.4). The response to tetanus toxoid and PPD was diminished with the addition of FCS to cultures compared to human serum. Human serum was therefore selected to support the *in vitro* proliferation assay.

4.1.5 Proliferation response in fresh and cryopreserved PBMC's

PBMC's are often cryopreserved in DMSO solution and stored in liquid nitrogen before use (see section 3.3.3). To determine if it was acceptable to use frozen cells in future assays experiments were performed to examine proliferative responses using fresh and cryopreserved PBMC's. Figure 4.5 compares the proliferative responses of PBMC's which

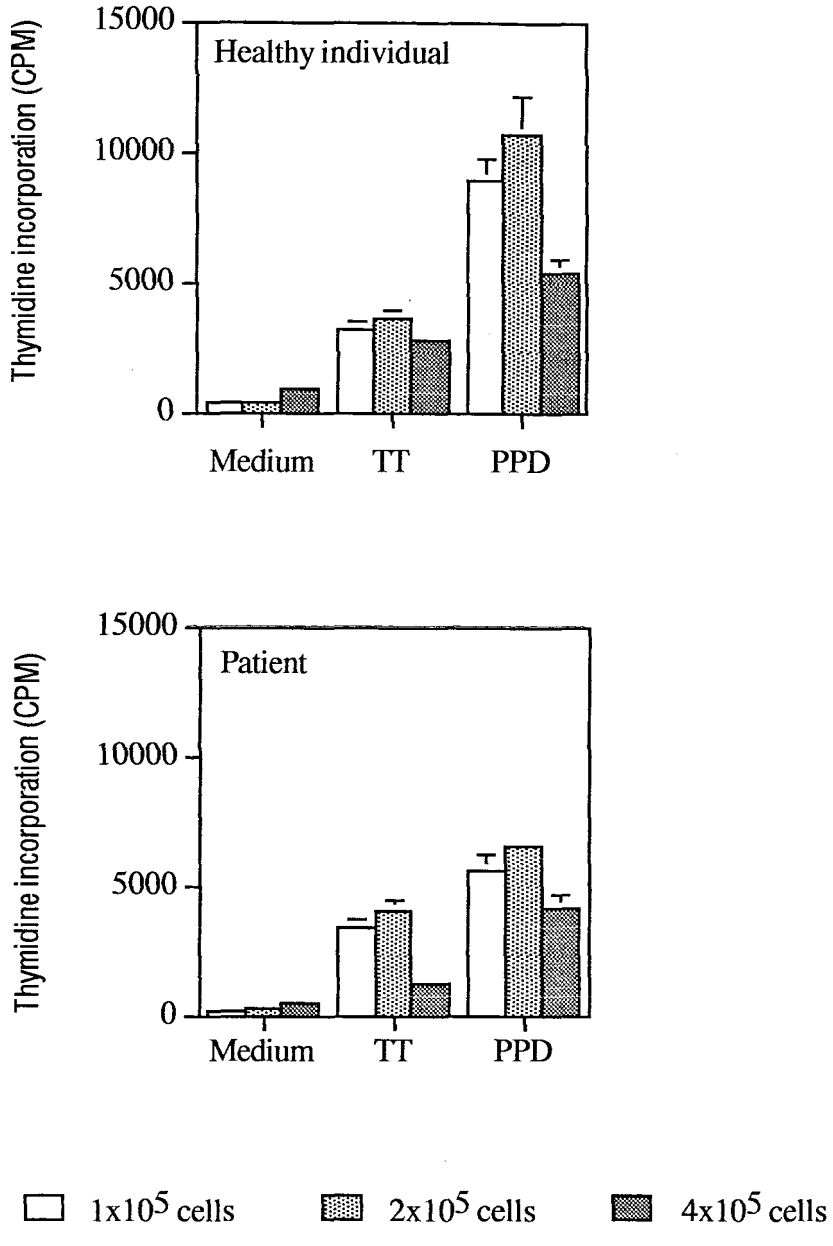


Figure 4.3

Proliferative responses (tritiated thymidine incorporation) obtained from PBMC's taken from a representative healthy individual and patient with SLE. Triplicate cultures of $1x10^5$, $2x10^5$ or $4x10^5$ cells per well were incubated for 7 days with either medium, tetanus toxoid or PPD (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

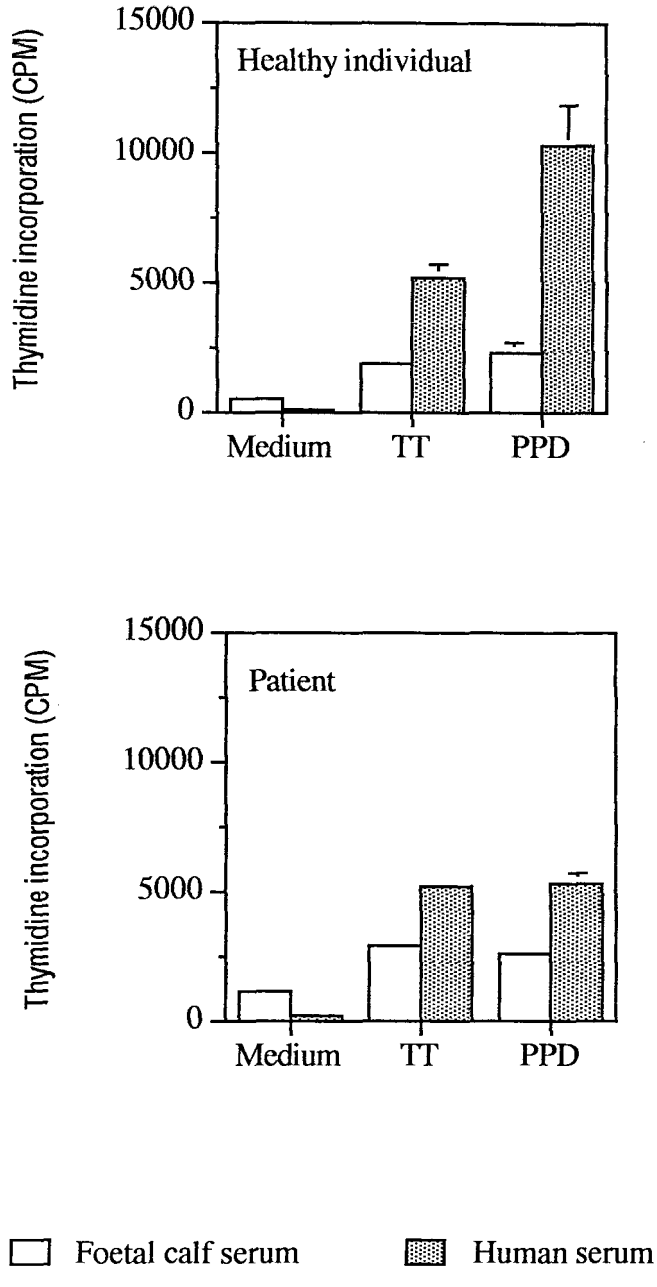


Figure 4.4

Proliferative responses (tritiated thymidine incorporation) obtained from PBMC's taken from a healthy individual and a patient with SLE. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with either medium, tetanus toxoid or PPD (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

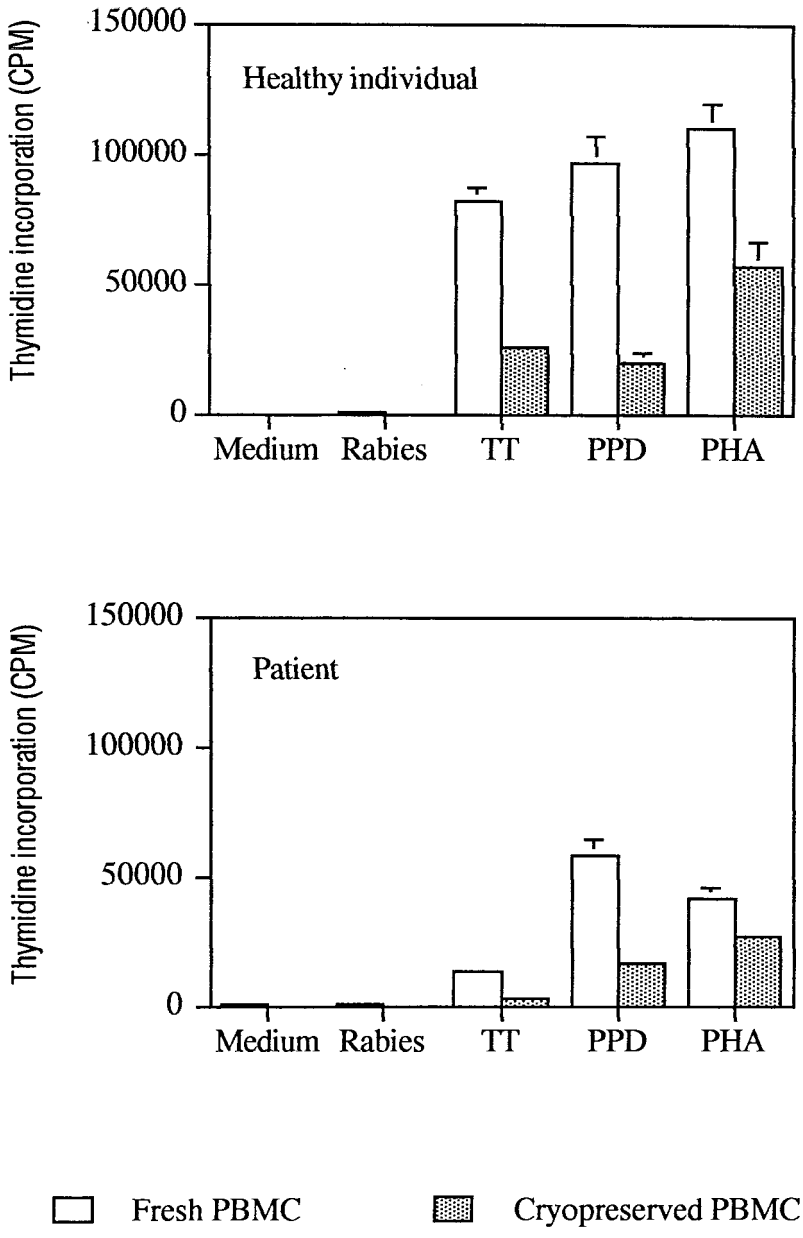


Figure 4.5

Proliferative responses (tritiated thymidine incorporation) obtained from PBMC's taken from a healthy individual and a patient with SLE using fresh or cryopreserved PBMC's. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with either medium, rabies, tetanus toxoid, PPD or PHA (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

had been freshly isolated and cryopreserved from the same effusion assayed two months later. For both the healthy individuals and patients the proliferative response was reduced when using cryopreserved cells. The reduced response was more relevant in the patients since they show a lower proliferative response to antigenic stimulation of fresh PBMC's compared to healthy individuals. It was therefore decided to use fresh PBMC's for this study.

4.1.6 Reproducibility of assay system

Using fresh PBMC's from two healthy individuals and two patients with SLE taken 6 months apart the quality of the assay system was further examined by studying the reproducibility of the responses. Figure 4.6 shows that the repeat assays are highly reproducible in response to stimulatory antigens. This demonstrates that results obtained over the period of study may be compared satisfactory using this assay system.

4.1.7 Summary of assay conditions

This section shows the development of a reproducible assay system for measuring proliferative responses to recall and naive antigens in healthy individuals and patients with SLE. On the basis of these findings a 7 day assay was adopted as standard. Fresh PBMC's were cultured at $2x10^5$ per well in RPMI supplemented with 10% heat inactivated human serum. Wells were pulsed for the final 18 hours of culture with tritiated thymidine. Antigens were used at the following concentrations; tetanus toxoid and PPD at 10μg/ml, rabies protein at 5μg/ml and PHA at 1μg/ml.

4.2 Improving the in vitro *proliferative response in patients with SLE*

It has been reported that the diminished proliferative responses observed in patients with SLE can be restored to that seen in healthy individuals by altering the culture conditions. This includes resting the cells before stimulation (Huang *et al.*, 1986), adding anti - CD28

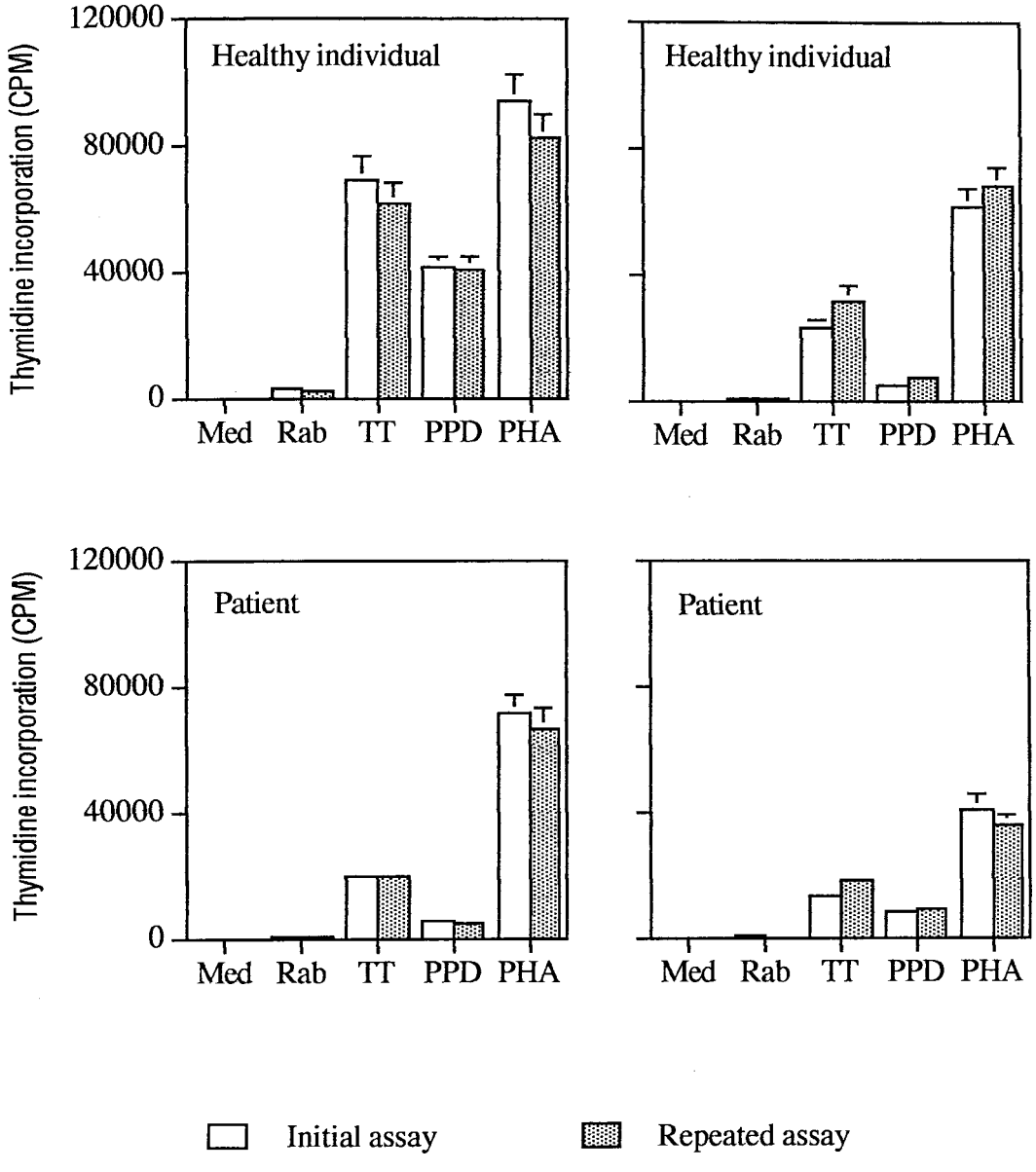


Figure 4.6

Proliferative responses (tritiated thymidine incorporation) obtained from fresh PBMC's taken from two healthy individuals and two patients with SLE. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with either medium, rabies protein, tetanus toxoid, PPD or PHA (see section 3.3.9). Assays were repeated six months apart. The results are expressed as mean CPM of the triplicates ± standard deviation.

monoclonal antibody (Alvarado *et al.*, 1994; Horwitz *et al.*, 1997) and removing the adherent cells from the culture (Linker-Israeli *et al.*, 1983; Fox *et al.*, 1991). In this section the aim was to assess some of these reported methods to determine if they would be beneficial to the present study. Fresh PBMC's were cultured with antigen plus anti - CD28 monoclonal antibody or were 'rested' for 5 days in fibroblast condition medium before being assayed. The results presented are from one healthy individual and one patient and are representative of the group as a whole.

4.2.1 Determining the optimum concentration of anti - CD28 monoclonal antibody

Fresh PBMC's were cultured for 7 days with tetanus toxoid or rabies protein and a range of concentrations of anti - CD28 monoclonal antibody. The antigens were selected to represent a recall response and a naive response. Proliferation was measured by tritiated thymidine incorporation during the final 18 hours of culture. Anti - CD28 monoclonal antibody inhibited the proliferative response to tetanus toxoid in PBMCs taken from healthy individuals (Figure 4.7). An anti - CD28 monoclonal antibody concentration of 0.05μg/ml had the least detrimental effect. The effect of the antibody on proliferation was not due to toxicity of the antibody since the viability did not alter between culture conditions (Figure 4.8). Inclusion of anti - CD28 monoclonal antibody had no effect on the proliferative response to tetanus toxoid in patients (Figure 4.7).

For the rabies protein, an increased proliferative response was observed in healthy individuals using 0.05μg/ml of antibody (Figure 4.7). At the other concentrations of antibody tested a reduced response was measured. Anti - CD28 antibody had no effect on the response to rabies protein in patients. From these data anti - CD28 antibody was used at a final concentration of 0.05μg/ml to examine the effect of anti - CD28 antibody on the proliferative responses to PPD and PHA.

4.2.2 Effect of anti - CD28 monoclonal antibody on proliferation

Proliferation assays were performed using PBMC's from healthy individuals and patients

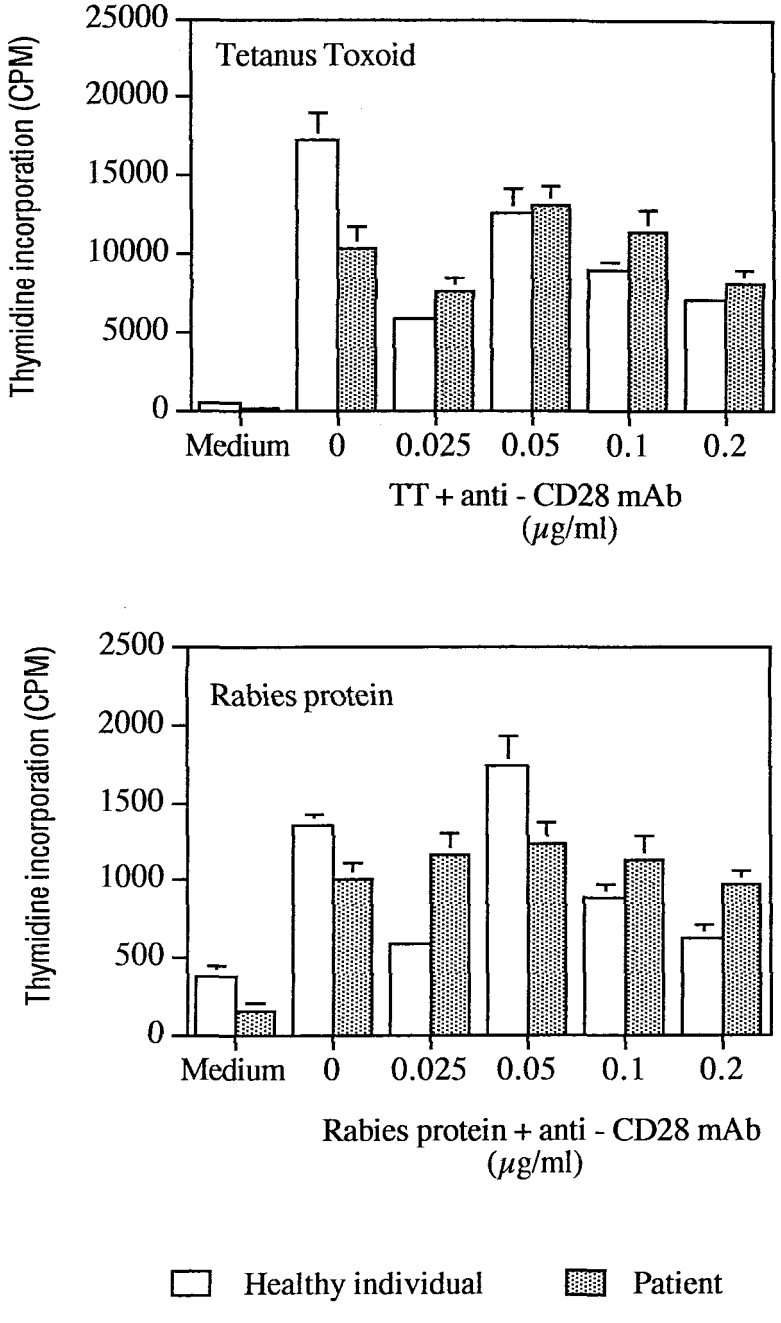


Figure 4.7

Effect of anti - CD28 monoclonal antibody on T cell proliferation in response to tetanus toxoid and rabies protein. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with anti - CD28 monoclonal antibody at 0.025μg/ml, 0.05μg/ml, 0.1μg/ml or 0.2μg/ml final concentration (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

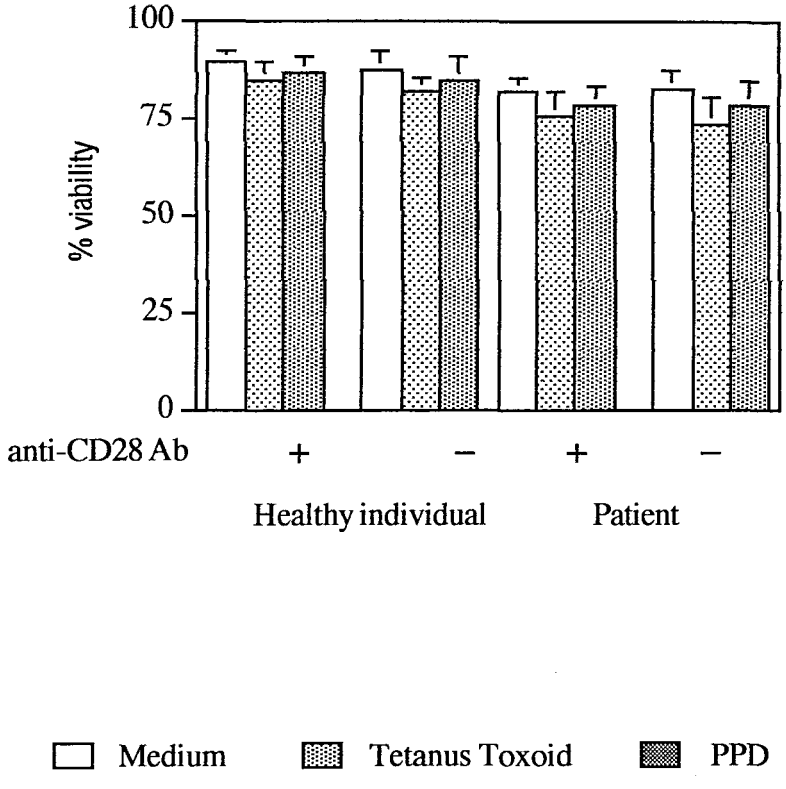


Figure 4.8

Percentage viability (as determined by trypan blue exclusion) in PBMC's from a healthy individual and a patient following stimulation with medium, tetanus toxoid or PPD at day 3 of culture in the presence and absence of anti - CD28 monoclonal antibody. Cultures were 100% viable on day 1. The results are expressed as mean CPM of the triplicates ± standard deviation.

with SLE in the presence of anti - CD28 antibody (Figure 4.9). The proliferative response to tetanus toxoid and rabies protein is described above (see section 4.2.1). The response to PPD and PHA was not affected by the addition of antibody to the cultures in healthy individuals or patients.

4.2.3 Effect of fibroblast condition medium on proliferation

Resting PBMC's from patients with SLE was reported to improve the proliferative response to antigen stimulation (Huang *et al.*, 1986). However, the cells are susceptible to spontaneous apoptosis. Since fibroblast condition medium (FCM) is reported to prevent spontaneous apoptosis (Akbar *et al.*, 1993) PBMC's were rested in RPMI supplemented with 50% FCM. Cells were rested for 5 days prior to stimulation with antigens. In healthy individuals the proliferative response was reduced to varying degrees after resting the cells in FCM (Figure 4.10). For some patients an enhanced proliferative response to stimuli after resting the cells in FCM was observed. In other patients resting the cells prior to stimulation appeared to have little effect (Figure 4.10). Since cell yields were reduced as a result of resting the cells and an enhanced response to stimuli did not occur in all of the patients tested it was decided not to include this step in the assay procedure.

4.2.4 Summary of restoring the cellular proliferative response

It has been reported that the reduced proliferative responses to standard antigens in patients with SLE could be restored using altered assay conditions. In this section of work the addition of anti - CD28 monoclonal antibody to the cultures and resting the cells in fibroblast condition medium prior to stimulation with antigen was considered.

Inclusion of anti - CD28 monoclonal antibody in the cell assay had no effect on the proliferative response in patients with SLE but slightly inhibited tetanus toxoid responses in healthy individuals. However, a 0.05μg/ml concentration of antibody improved the proliferative response to rabies protein in healthy individuals. This is most likely due to naive T cells requiring more stringent costimulatory signals compared with primed T cells.

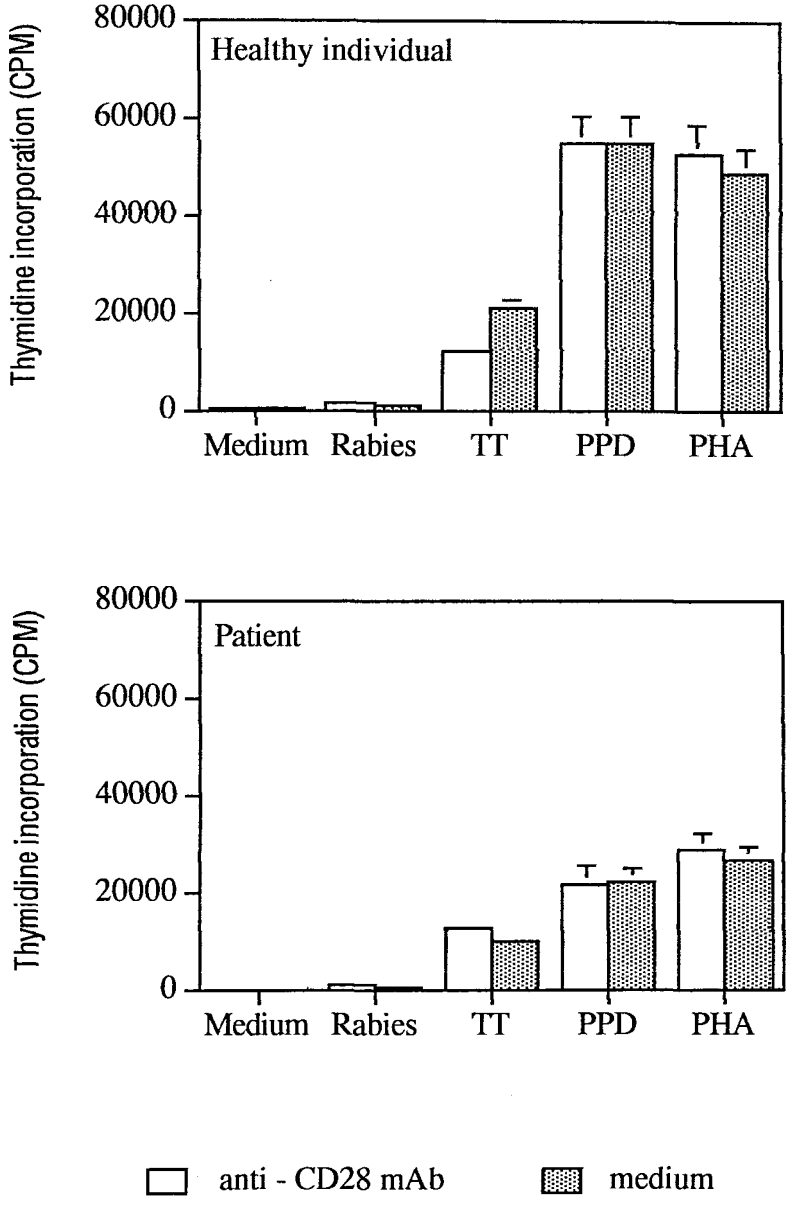


Figure 4.9

Effect of anti - CD28 monoclonal antibody on T cell proliferation in response to rabies, tetanus toxoid, PPD and PHA in a healthy individual and a patient with SLE. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with or without anti - CD28 antibody (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

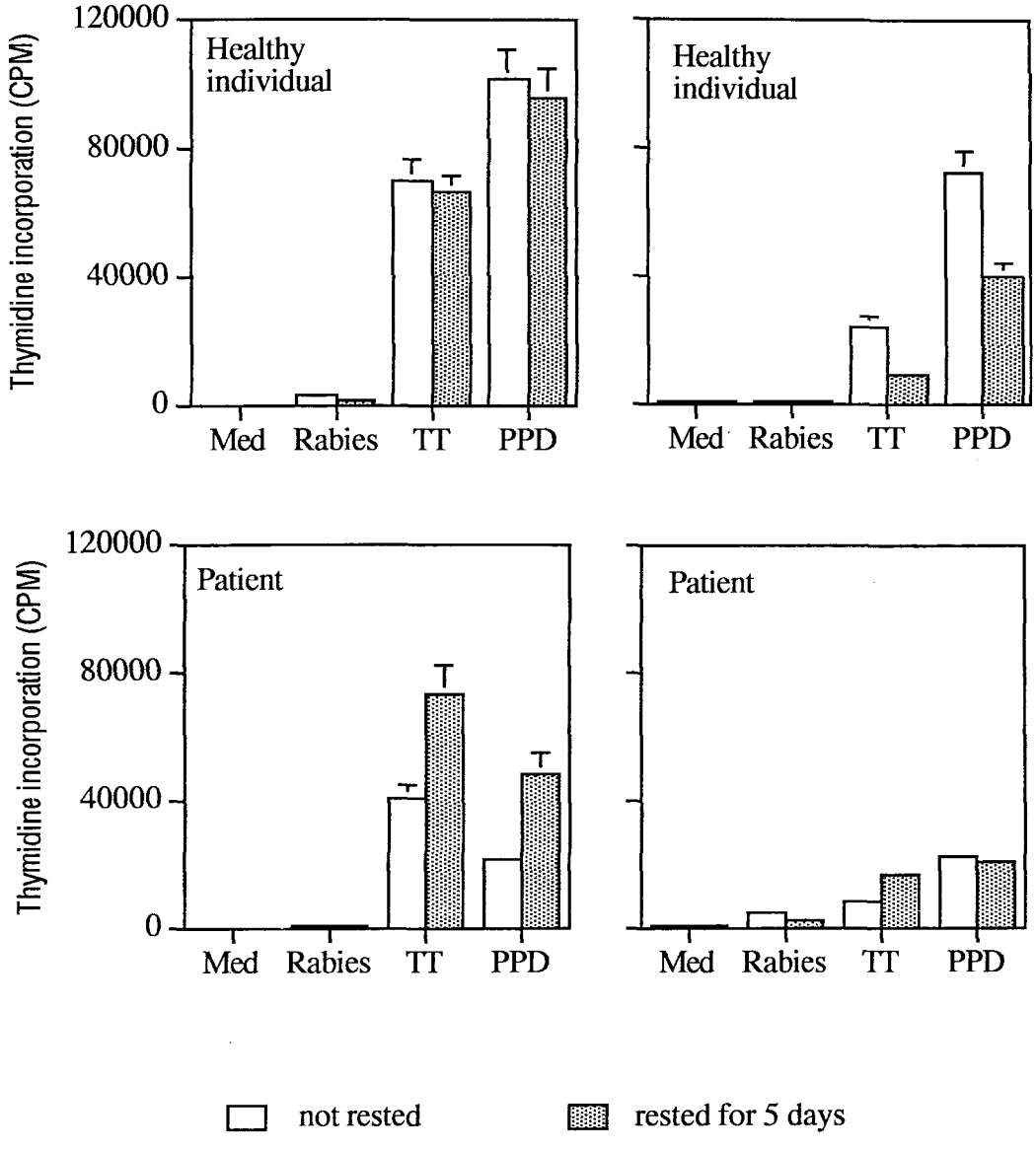


Figure 4.10

Effect of resting PBMC's in fibroblast condition medium for 5 days prior to stimulation with rabies protein, tetanus toxoid or PPD in two healthy individuals and two patients with SLE. Open bars represent proliferation of cells which have not been rested and dotted bars represent cells which have been rested in FCM. Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with antigen and anti - CD28 monoclonal antibody (see section 3.3.9). The results are expressed as mean CPM of the triplicates ± standard deviation.

Since the activation requirements of autoreactive cells is poorly understood, and anti - CD28 antibody had no detrimental effects on responses in patients, the antibody was not removed from the assay. Anti - CD28 antibody was used at 0.05μg/ml in all proliferation assays, unless otherwise stated, in order to optimise the detection of autoreactive T cell responses.

Resting PBMC's in FCM prior to stimulation improved proliferative responses to antigenic stimulation in only a few patients. However, since the cell yields were reduced following the resting period it was decided not to 'rest' the PBMC's prior to stimulation with antigen.

4.3 Proliferative response to previously encountered antigens

The proliferative response to autoantigens forms the main thrust of this work. Responses to purified β_2 glycoprotein 1 is examined in Chapter 5 and the response to synthetic peptides of the La protein is examined in Chapter 6. However, O'Keeffe *et al.* (1999) report that the response to PPD is much greater than gliadin, an autoantigen in coeliac disease, in healthy individuals and patients and Reece *et al.* (1994) report that the number of cells responding to tetanus toxoid is greater than to synthetic peptides of tetanus toxoid. It was therefore considered necessary for the identification of autoreactive cells in the later studies to identify patients with SLE who showed a good response to recall and naive antigens. A criticism of published work relating to responses to recall antigens in SLE is the failure to state if the patients have been exposed to the stimuli previously. Additionally, vaccinations have been implicated in the induction of SLE resulting in some reluctance to immunise patients. The aim of this section was therefore to compare the vaccination status with the proliferative response and to determine if the ability to respond to one antigen prejudices the ability to respond to another.

4.3.1 Proliferative responses to standard antigens in healthy individuals and patients

The proliferative response to antigenic stimuli was compared between 35 healthy individuals (27 females and 8 males; age range 27 - 59 years) and 99 patients with SLE (93 females and 6 males; age range 20 - 76 years). CPM values for each subject are given in Appendix A. The proliferative response for the group of healthy individuals and patients to tetanus toxoid are given in Table 4.1. There was a statistically significant difference (Student's T Test) between healthy individuals and patients in the proliferative response to tetanus toxoid ($p = 0.007$), PPD ($p = 0.0001$), rabies protein ($p = 0.03$) and PHA ($p = 0.0002$). There was no significant difference in the average background proliferation between patients and healthy individuals ($p = 0.97$).

Table 4.2 summaries the number of patients capable of mounting an *in vitro* response to each of the standard antigens studied (tetanus toxoid, PPD, rabies protein). A stimulation index of three was used to determine a positive response. In summary, all of the healthy individuals responded to at least one of the antigens while nine patients failed to respond to any of the antigens. A greater proportion of patients responded to only one antigen compared to the healthy individuals. A similar proportion of healthy individuals and patients responded to two antigens. Hence, a greater proportion of healthy individuals responded to all three antigens. The ability to respond to stimuli *in vitro* was not a reflection of severity of disease nor medication. Seventy one patients were taking < 20mg prednisone per day while the remaining twenty eight were not receiving medication. All of the healthy individuals and patients responded to PHA.

4.3.2 Tetanus toxin vaccination

One explanation for the difference in proliferative response between healthy individuals and patients to tetanus toxoid may be the number of individuals vaccinated with tetanus toxin. The proliferative responses to tetanus toxoid were therefore compared to the vaccination status. All of the healthy individuals (8 males and 27 females who covered the age range of 27 - 59) had been vaccinated against tetanus toxoid. Seven had been

Antigen	Vaccination	Healthy individuals		Patients	
	Status	No.	CPM ± sd	No.	CPM ± sd
Tetanus	—	35	26128 ± 30385	99	10867 ± 15485
toxoid	< 10 years	28	30852 ± 32485	50	15864 ± 18694
	> 10 years	7	14626 ± 14450	37	6551 ± 9787
	never	0	—	12	3809 ± 5357
PPD	—	35	36955 ± 32767	99	12575 ± 15408
	Yes	35	36955 ± 32767	78	13706 ± 16543
	No	0	—	21	8102 ± 9184
Rabies	—	35	1598 ± 1419	99	1004 ± 1092
PHA	—	35	62541 ± 35166	99	36124 ± 25844
Medium	—	35	513 ± 249	99	479 ± 267

Table 4.1

Comparison of the mean proliferative response (and standard deviation) in 35 healthy individuals and 99 patients with SLE to tetanus toxoid, PPD, rabies protein, PHA and medium. Proliferative responses were also compared between subjects grouped according to vaccination status; vaccinated within the last 10 years (< 10 years), longer than 10 years (> 10 years) or never vaccinated. — denotes that the vaccination status was not used to compare proliferative responses.

Antigen	Healthy individuals		Patients	
	No.	(%)	No.	(%)
None	0	(0)	9	(9)
Tetanus toxoid only	0	(0)	6	(6)
PPD only	2	(6)	26	(26)
Rabies protein only	0	(0)	1	(1)
TT + PPD	18	(51)	42	(42)
TT + Rabies protein	0	(0)	1	(1)
PPD + Rabies protein	1	(3)	3	(3)
TT + PPD + Rabies	14	(40)	11	(11)
n =	35		99	

Table 4.2

Number (and percentage) of healthy individuals and patients responding to antigenic stimuli *in vitro*. $2x10^5$ fresh PBMC's were cultured with either tetanus toxoid, PPD or rabies protein and anti - CD28 monoclonal antibody for 7 days. Proliferation was determined by tritiated thymidine incorporation during the final 18 hours of culture (see section 3.3.9).

vaccinated greater than 10 years ago while 28 had been vaccinated within the last 10 years. Of the patients, 6 were male and 93 female and covered the age range 20 - 76. Twelve patients had never been vaccinated with tetanus toxoid, 37 had received the vaccine greater than 10 years ago and 50 had received the vaccine within the last 10 years (see Appendix A).

Table 4.1 summaries the proliferative responses to tetanus toxoid in healthy individuals and patients. A full set of data is given in Appendix A. Healthy individuals who had never been vaccinated with tetanus toxoid were not available for this study although a search for such individuals was made with the aid of the West Midlands Blood Transfusion Centre. There was a statistically significant difference (Student's T Test) between healthy individuals and patients vaccinated within the last 10 years ($p = 0.03$) but not when individuals were vaccinated greater than ten years ago ($p = 0.33$). There was no statistically significant difference (Student's T Test) between healthy individuals vaccinated within the last ten years compared with healthy individuals vaccinated greater than ten years ago ($p = 0.117$). However, in patients there was a significant difference between vaccination within the the last ten years compared with vaccination greater than ten years ago ($p = 0.004$). There was no significant difference between patients vaccinated greater than ten years and never vaccinated ($p = 0.21$). The range of proliferative responses to tetanus toxoid between healthy individuals and patients for each vaccination group was similar. It was not possible to determine if a subject had been vaccinated in the last year or nine years ago but such subjects would be categorised into the same group. This may partially explain the variation within each group. However, a natural variation exists in the ability of subjects to respond to antigen.

There was no significant difference in the background proliferative responses between healthy individuals and patients. This was irrespective of vaccination status. The average background proliferative responses in healthy individuals was 506 ± 262 CPM and 552 ± 155 CPM for subjects vaccinated within the last ten years and greater than ten years ago, respectively. The average background proliferative responses in patients was 514 ± 267 CPM, 474 ± 288 CPM and 552 ± 155 CPM for subjects vaccinated within the last ten

years, greater than ten years ago and never vaccinated, respectively.

In order to determine if vaccination before and after clinical diagnosis affects the *in vitro* proliferative response patients who had been clinically diagnosed in the last ten years but vaccinated greater than 10 years ago were studied. The proliferative response in these patients was compared to the proliferative response of patients diagnosed greater than 10 years ago but vaccinated within the last 10 years. In patients vaccinated before clinical diagnosis (n = 16) the response to medium was 603 ± 313 CPM (192 - 982 CPM) and the response to tetanus toxoid was 10221 ± 11851 CPM (124 - 39677 CPM). In patients vaccinated after clinical diagnosis (n = 33) the response to medium was 492 ± 309 CPM (106 - 1515 CPM) and the response to tetanus toxoid was 14980 ± 17567 CPM (223 - 61674 CPM). Vaccination before and after clinical diagnosis had no statistically significant effect on the proliferative response to tetanus toxoid (medium $p = 0.25$; tetanus toxoid $p = 0.27$).

Sera from the healthy individuals (n = 35) and patients (n = 99) were tested for anti - tetanus toxoid IgG antibodies and IgG antibodies using specific ELISA's (Table 4.3). There was no significant difference in the IgG or anti - tetanus toxoid antibody titres between healthy individuals and patients with the same or different vaccination status.

In summary, the mean proliferative response to tetanus toxoid in healthy individuals was greater than in patients for each vaccination group. The mean proliferative response decreased for both healthy individuals and patients as the length between examining the *in vitro* proliferative response and vaccination increased. However, the range of proliferative responses between healthy individuals and patients was similar. Serum IgG levels were greater in patients than healthy individuals irrespective of vaccination status. Anti - tetanus toxoid titres were equivalent in healthy individuals and patients when vaccinated within the last ten years. Antibody titres were maintained by healthy individuals, but not patients, vaccinated greater than ten years ago.

Vaccination	Healthy individuals		Patients	
status	IgG	TT Ab	IgG	TT Ab
< 10 years	15.3	89	18.8	90
	(8.7 - 22.7)	(5 - 141.6)	(10 - 42.4)	(3 - 141)
> 10 years	12.8	80.8	18.9	57.4
	(7.6 - 19.8)	(49.4 - 128.8)	(3.4 - 41.3)	(0 - 136)
Never	—	—	19.2	31.4
			(10 - 31.7)	(0 - 130)

Table 4.3

Serum IgG and anti - tetanus toxoid antibody titres in healthy individuals and patients. Subjects were divided into three groups according to the vaccination status with tetanus toxoid; vaccinated within the last ten years (< 10 years), greater than 10 years (> 10 years) or never vaccinated (never). The mean titre is given with the range of titre in brackets below.

4.3.3 BCG vaccination

BCG is a vaccine given to protect against infection with *Mycobacterium tuberculosis* and contains live, attenuated *Mycobacterium bovis*. Purified protein derivative of tuberculin (PPD) is an extract of mycobacteria and is used in the Mantoux reaction to determine exposure to the bacteria. To determine if the difference in the proliferative response to PPD between healthy individuals and patients was due to previous exposure the proliferative response to PPD was compared with the BCG vaccination status. All of the 35 healthy individuals had received the BCG vaccine. Of the 99 patients studied 78 had received the BCG vaccine while 21 had not. Table 4.1 summaries the proliferative response to PPD *in vitro*. A full set of data is given in Appendix A. There was a statistically significant difference in the proliferative response to PPD between healthy individuals and patients vaccinated with BCG ($p = 0.0003$). There was a significant difference in the response between patients who had been vaccinated and those who had not ($p = 0.046$). However, it cannot be excluded that some patients who had not been vaccinated had been exposed to the antigen.

In summary, patients previously exposed to antigen showed a reduced proliferative response *in vitro* to stimuli compared to healthy individuals. However, antibody titres were similar in healthy individuals and patients. These data however do not attempt to explain why a reduced proliferative response is observed in patients. However, there was a wide range of proliferative responses in all groups studied, with some patients showing a greater response to antigen compared with the healthy individuals.

4.4 Discussion

4.4.1 General features of the proliferation assay

Antigen specific CD4 T cells require stimulation *in vitro* before their existence can be determined using the current methods available. In this study a highly reproducible cell

assay was developed to measure cellular responses to tetanus toxoid, PPD and rabies protein. Fresh PBMC's were cultured with optimal antigen concentrations and anti - CD28 monoclonal antibody. Proliferation was determined by tritiated thymidine uptake during the final 18 hours of culture (see section 3.3.9). Fresh PBMC's were used since a reduced proliferative response was measured when using cryopreserved cells. The reduction was greater in patients than in healthy individuals and may be due to the increased number of active T cells in patients with SLE which are lost upon cryopreservation. Optimal proliferation was observed on day 7 for all of the antigens studied (Figure 4.2). However, it cannot be excluded that the optimal proliferative response occurred on day 6 or 8 since cultures were not harvested on these days. Barker and Elson (1994) reported that memory responses are optimal on day 6 while naive responses are optimal on day 8. Assays should have therefore been harvested every day between 5 and 7 days in order to examine more thoroughly the kinetics of the responses.

Human serum was selected to maintain the cultures since a higher background proliferative response was measured with foetal calf serum compared to human AB serum (Figure 4.4). This is in agreement with Rumley *et al.* (1984) and can be explained by the presence of bioactive components in the FCS which are mitogenic to human cells. Rumley *et al.* (1984) recommend using human albumin to support cell cultures since it supports low background responses and cultivates good responses to antigenic stimuli. Human albumin was not tested in the present study. However, transferrin is a major component of serum and is required for cell division. Gaston *et al.* (1987) showed that T cells respond equally well to transferrin or human serum supplemented medium. Under normal circumstances CD4+ T cells may be able to produce sufficient transferrin to maintain the culture. Since this project is focused on responses in PBMC's from patients with SLE in which proliferation *in vitro* is reduced (see section 4.3), human serum was selected as the source of nutrients. Human serum contains all of the essential factors required for supporting cell proliferation and therefore should not be a limiting factor.

4.4.2 Patients with SLE show a reduced proliferative response to standard antigens

Studies examining *in vitro* T cell proliferative responses in patients with SLE yield conflicting data (see section 1.3.5). However, there is a consensus that PBMC's from patients with SLE show a poor proliferative response to PHA stimulation (Fox *et al.*, 1991; Stekman *et al.*, 1991; Utsinger and Yount, 1977). This finding was confirmed in the present studies (see section 4.3.1). PHA activates cells via the CD2 surface molecule independently of accessory signals. This suggests that there is a defect in the CD2 pathway in SLE. Fox *et al.* (1991) showed a reduced proliferative response to anti - CD2 antibody by patients with SLE which did not correlate with a decreased expression of either CD2 molecules or IL2 receptor. In addition, it has been reported that complement deficient patients (Utsinger and Yount, 1977) and those with active disease (Hughes *et al.*, 1976) exhibit lower responses to PHA. These patients are more likely to be on intensive steroid treatment which may impair cellular proliferative responses. However, the present study did not identify any correlation between the ability to proliferate in response to PHA and renal disease (a marker of disease severity) or medication.

The proliferative response to anti - CD3 antibody has also been studied in SLE. Stekman *et al.* (1991) showed that PBMC's from patients with SLE elicit a better response, compared to healthy individuals, to anti - CD3 antibody crosslinked with anti - mouse IgG. The CD3 pathway is dependent on the interaction with IL2 and the IL2 receptor but Blasini *et al.* (1994) showed that IL2 production and IL2 receptor expression were equivalent in patients and healthy individuals before and after stimulation with anti - CD3 antibody. In contrast to the effect of anti - CD3 antibody, a reduced response to tetanus toxoid and PPD, which use the TCR / CD3 pathway to activate T cells, has been demonstrated in the present study. This is in agreement with Gottlieb *et al.* (1979), Fox *et al.* (1991) and Tsokos *et al.* (1996). The poor response was not due to suboptimum antigen concentration. However, some patients with SLE showed a better proliferative response to the recall antigens compared to some healthy individuals. A natural variation in the ability to proliferate in response to antigenic stimulation exists between individuals suggesting that the T cell response in some patients with SLE is normal. Alternatively, it suggests that the patients

but not healthy individuals have been exposed to the antigens previously. However, all of the healthy individuals enrolled on to the study had been vaccinated with tetanus toxin.

4.4.3 Restoring the proliferative response in patients with SLE

Antigen specific activation of T cells requires two signals, one through the TCR and a second through a costimulatory molecule in the form of CD28 and B7 interaction (see section 1.1.3). Failure to receive both signals results in T cell anergy. This suggests that a lack of costimulation may be responsible for the reduced T cell response to antigens in SLE PBMC's. To test this hypothesis anti - CD28 monoclonal antibody was added to the cell cultures. Anti - CD28 antibody inhibited the response to tetanus toxoid in healthy individuals (Figure 4.9). This is in agreement with Yi-qun *et al.* (1996). In addition, Damle *et al.* (1991) reported that the proliferative response to PPD by CD4+ CD45RO+ T cells was suppressed by anti - CD28 antibody. However, memory T cell responses are not dependent on the CD28 pathway therefore anti - CD28 antibody should not have an effect on proliferation to recall antigens (Lovett-Racke *et al.*, 1998). In the published studies it is possible that anti - CD28 antibody is having an antagonistic effect (Yi-qun *et al.*, 1996; Damle *et al.*, 1991). However, in the present study PHA and PPD responses were not affected by anti - CD28 antibody and there was no difference in cell viability between cultures with and without antibody. The response to rabies protein was enhanced by anti - CD28 antibody in healthy individuals confirming that additional signals through the CD28 pathway can enhance a naive T cell response.

In patients, inclusion of anti - CD28 antibody in the cell assay had no effect on the proliferative response to the antigens studied (Figure 4.9). This is in contrast to published reports showing that anti - CD28 antibody improves the already enhanced proliferative response to anti - CD3 antibody in patients with SLE (Kaneko *et al.*, 1996; Alvarado *et al.*, 1994; Sfikakis *et al.*, 1994). One explanation for the different findings is that anti - CD3 antibody is an artificial method for cell stimulation providing excess signals into the cell. Biological relevant antigens may be unable to generate the necessary cell signalling in SLE PBMC's, perhaps through poor processing and presentation, and anti - CD28

antibody is insufficient to compensate for this and restore proliferation. In addition, there is a reduced number of CD28+ T cells (Kaneko *et al.*, 1996), and possibly a lower T cell frequency specific for tetanus toxoid, in patients with SLE. Furthermore, Garcia-Cozar *et al.* (1996) report that macrophages from patients with SLE fail to show increased expression of B7 following PHA stimulation. Sfikakis *et al.* (1994) showed that B7.1 transfected P815 mouse cells significantly enhanced the response to tetanus toxoid when cultured with SLE PBMCs but not with PBMCs from healthy individuals. This implies that CD28 signalling is not impaired in patients with SLE. In the present study anti - CD28 antibody was added to the cultures in a soluble form and binding it to the plate may enhance the signals delivered to the cell and improve the proliferative responses.

It is speculated that the poor T cell response to recall antigens in patients with SLE is due to the presence of highly activated cells which are refractory to further stimulation *in vitro*. Hence, the effect of resting the cells prior to stimulation has been examined. In the present study resting the cells did not restore proliferative responses to recall or naive antigens. This is in agreement with Fox *et al.* (1991) who reported that resting cells for 24 - 48 hours failed to improve the response to anti - CD2 antibody in patients with SLE. In contrast to these findings, Huang *et al.* (1986) showed that IL2 production is restored to normal levels following PHA and PMA stimulation only if the cells are rested for 3 days prior to stimulation. The effect on cell proliferation was not reported. In contrast, Volk and Diamantstein (1986) report that IL2 restores tetanus toxoid responses in patients with SLE. In addition, Warrington *et al.* (1989) reported that resting the cells increased the precursor cell frequency responding to IL2 but that IL2 produced during the resting period was not responsible for any improved responses.

Linker-Israeli *et al.* (1983) showed that removing adherent cells (mainly macrophages) prior to antigenic stimulation improved the production of IL2 in patients with SLE. Additionally, Sasaki *et al.* (1989) reported that macrophage depleted PBMC cultures produce lower titres of anti - DNA antibodies following stimulation with PWM. However, in the present study resting the cells resulted in the removal of adherent cells since cells were rested in 24 well tissue culture plates. Non adherent cells were then transferred to 96

well plates and stimulated with antigen. However, removing the adherent cell population did not affect the proliferative response although the adherence step is insufficient to remove all of the macrophages. One possible explanation for the findings of the present study is that recall antigens were not presented in sufficient quantity since they require APC's for processing and presentation.

4.4.4 Vaccination status reflects proliferative responses to recall antigens

The present study, and Gottlieb *et al.* (1979), show that proliferative responses to tetanus toxoid are lower in patients than in healthy individuals. However, T cell responses, as measured in the present study, are similar in healthy individuals vaccinated greater than ten years ago and patients vaccinated within the last ten years (14626 CPM and 15864 CPM respectively). Additionally, vaccination before and after clinical diagnosis did not effect the proliferative response to tetanus toxoid *in vitro*. This is in agreement with Gottlieb *et al.* (1979). Gottlieb *et al.* (1979) report that the reduced proliferative response is due to a T cell defect and not a defect in the macrophage population. In addition, both studies (this thesis and Gottlieb *et al.*, 1979) report that serum anti - tetanus toxoid antibody titres are similar in healthy individuals and patients. However, antibody titres are maintained in healthy individuals but not in patients. The data suggests that patients are unable to produce and maintain a sufficient number of functional memory T cells to maintain antibody production.

This thesis and Gottlieb *et al.* (1979) fail to explain the relationship between the poor proliferative response to tetanus toxoid but normal serum antibody titre in patients. Nies *et al.* (1980) show through co - culture experiments that the anti - tetanus toxoid antibody titre is reduced in patients with SLE. This reduction was due to a lack of B cell help and not an abnormality in the T cell. Nies *et al.* (1980) speculated that some type of antibody feedback suppression was taking place in SLE patients. However, serum from patients with SLE often has increased antibody titres implying that any feedback mechanism was specific to the antigen. Alternatively, the contradictory antibody titres found in serum compared to *in vitro* studies may be due to an artifact of the assay system.

In conclusion, an assay system was developed to measure proliferative responses in patients with SLE to naive and recall antigens. The assay system was then applied to measuring proliferative responses to potential autoantigens (β_2 glycoprotein 1 and La protein) in SLE, as examined in the following chapters.

Chapter 5

Results Section 2

β_2 Glycoprotein 1 as a T cell autoantigen

Anti - phospholipid antibodies (APA), which include lupus anticoagulant (LAC) and anticardiolipin antibodies (ACL), are found in the sera of patients with anti - phospholipid syndrome (APS) and SLE (Roubey, 1996). These autoantibodies are associated with arterial and venous thrombosis, recurrent foetal loss, livedo reticularis and thrombocytopenia (Alarcon-Segovia, 1992). Primary APS, first described by Alarcon-Segovia and Sanchez-Guerrero (1989), is defined as the presence of LAC and ACL in the absence of the clinical features associated with SLE.

Recent data indicates that APA's require the presence of protein cofactors such as β_2 glycoprotein 1 (β_2GP1), prothrombin, protein S and protein C for binding to phospholipids (McNeil *et al.,* 1990; Tsutsumi *et al.,* 1996; Fleck *et al.*, 1988; Oosting *et al.*, 1993). The antibodies are IgG class switched, show somatic hypermutations and are associated with the HLA DR4 haplotype (Loizou *et al.*, 1992; Arvieux *et al.*, 1994; Arnett *et al.*, 1991; McHugh and Maddison, 1989). This suggests that the antibody production is an antigen driven T cell dependent mechanism requiring antigen presentation by an APC. However, there is no evidence that lipids bind to the MHC molecule or to the TCR and that this interaction leads to T cell activation. Lipids can bind CD1 molecules, which have a similar structure to the MHC molecule, but how this lipid complex could activate T cells is unknown (Zeng *et al.*, 1997). Since APA's require protein cofactors for binding to phospholipids it has been proposed that an APA protein cofactor acts as the T cell autoantigen to drive the response.

In addition to the presence of serum ACL and LAC some patients with SLE have serum antibodies to the phospholipids phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Drouvalakis and Buchanan, 1998). PE, PS and PC are cell

membrane phospholipids which are exposed in early apoptosis (Savill *et al.*, 1993; Fadok *et al.*, 1992). Manfredi *et al.* (1998a) showed that APA's bind apoptotic cell membranes which leads to increased phagocytosis. Additionally, APA specifically bind β2GP1, which binds to negatively charged surfaces of apoptotic cells, suggesting that the clearance of apoptotic cells occurs via a β2GP1 dependent pathway (Manfredi *et al.*, 1998b). APL and anti - β2GP1 antibodies may prevent this clearance and hence lead to an excess of apoptotic material available as a source of antigen. This hypothesis is supported by reports describing T cell responses to nucleosomes in patients with SLE (Voll *et al.*, 1997b).

From the published data β2GP1 is the most attractive of the APA protein cofactors to initiate the generation of antibodies to phospholipids. This chapter reports the use of a standard T cell proliferation assay to determine if β2GP1 is a T cell autoantigen in APS or SLE and if β2GP1 specific T cells are associated with APA's or anti - β2GP1 antibodies.

5.1 Subjects

Fifty six Caucasian patients with SLE and / or APS, covering the age range 23 - 65, were enrolled into the study (Table 5.1). Sera from all of the patients was analysed for LAC, ACL and anti - β2GP1 antibodies (see section 3.2 and 5.2). The clinical features of each patient was assessed by Dr C. Gordon and Dr. S. Bowman. Thirty two of the 56 patients had clinical and / or serological features of APS. Fifteen of these 32 patients had SLE with APS or primary APS and high titres of serum anti - β2GP1 antibodies - 'APS + β2GP1'. The remaining 17 patients had SLE with APS and no, or very weak, serum anti - β2GP1 antibodies - 'APS'. Twenty four of the 56 patients had SLE without the clinical features of APS and no serum anti - β2GP1 antibodies. The terms 'APS + β2GP1', 'APS' and 'SLE' have been used throughout this thesis to differentiate between the groups of patients.

Sixteen healthy individuals, covering the age range 26 - 52 were enrolled into the study. A number of non - lab staff were included to avoid the possibility of accidental exposure to the antigen preparations used. None of the healthy individuals had LAC, ACL or anti -

Table 5.1

	D.O.B.	Symptoms	Medication	HLA DR	HLA DQ
APS + ß2GP1					
KA	21.6.76	T, R	P, A	0101, 0401-22	05, 0301/4
ACS	25.2.45	D	W	1301, 0401	0603, 0301/4
NH	16.5.69	M	P	0304, 1101/4/6	02, 0301
RHU	17.12.69	M		0701, 1301	0201/2, 0603
SH	6.9.46		P, M	0401/4	0301/4, 0302
AJ	18.7.68	R, M, A, H	P, A	0404, 0301	0201/2, 0302
LJ	19.2.64	M	P, A	0101/2/4, 0401	05, 0302
PM	7.12.67	R, H	H, P, A	0401, 0304	02, 0302
MN	15.4.79	M, D, E	W	0301, 1302	0604-9, 0201/2
GP	1.5.56	R, M, A, H, C	H, P, M, C, W	1001, 1302	05, 0604-9
JPI	22.6.62	M, A	W	1501, 0401-22	0602, 0302
MR*	12.11.49	C, L		0301, 0401-22	0301/4, 0201/2
SS	29.11.52	A	W	0401-22	*0302
TW	19.12.66	M, D, P, E	W	1501, 1301	0601, 0603
LY	30.1.60	I	P	0401, 0701	02, 0601/2
APS					
AB	20.5.49	M, T	P, C	1501, 0301	0201/2, 0602
KB	27.8.71	P	P, A, W	*0301	*0201/2
HCO	28.6.73	T, P	H, P, A, W	0101, 0301	0201/2, 0501-4
JF	21.7.43	M, D, P	P, W	0401, 1302	0604-9, 0301/4
RK	27.11.53	M, D, P	P, A, W	0401, 0301	0201/2, 0301/4
KL	1.1.60	D, A, M		0402, 13	0301/4, 0302
MH	10.11.35	R, A, S	P, A	0701, 1401/7	0201/2, 0501-4
CO	13.4.47	C	W	0301, 0701	*02
JOR	23.2.43	A, T	H, P	1501, 1401/7	0501-4, 0601/2
DP	1.8.49	R, A, L, P	P, W	11, 0301	0201/2, 0301/4
APO	26.6.44	M	H, P	0301, 1302	0201/2, 0604-9
RS	16.8.68	M, D	H, P	0103, 0901	0201/2, 0501-4
SUS	14.7.49	M, A, C	P, M	*0301	0201/2
AT	17.9.51	M, T, I, D, P	A	0301, 0701	0201/2
LV	4.9.47		H, P	0301, 11	0201/2, 0301/4
JW	11.7.63	R, T, D	H, P, A	*0304	*02
NW	20.11.59	M, D	P, W	0301, 0701	0201/2

Table continued overleaf

Table 5.1 continued

	D.O.B.	Symptoms	Medication	HLA DR	HLA DQ
SLE					
JA	28.6.42		H, P, A	1301, 1303	0603, 0301/4
SA	1.8.45			1601-6, 0301	0201/2, 0501-4
CB	25.12.37			1501, 0401	0602, 0301/4
JB	13.6.56			1501-5, 0301	0601/2, 02
MB	17.10.41	T	H, P, A	1501, 0301	0201/2, 0602
MBO*	15.1.46			0701, 1302	0201/2, 0604-9
AD	1.3.75	R	P, C	1501-5, 0901	*06, 09
KD	21.5.64			0101, 0401	0501-4, 0301/4
MDR	22.1.33			0101, 0301	0201/2, 0501-4
AF	17.3.48		H	1501-5, 0801-11	04, 0602
CF	13.3.64		P, C	1501	*0602
BGR	10.7.47		P	0101, 0301	0201/2, 0501-4
KH	20.1.69	R	P	0701, 1501	0303, 0602
RH	13.3.73	R, T	H, P, A	*0901, 0304	02, 0303
HJ	18.12.73		P, A	1501, 0301	0201/2, 0602
PK	14.7.48		H, P	1301, 0301	0201/2, 0603
HM	1.12.49		H, P	*0301	*02, 02
HMO	28.2.66		P, A, W	1501	*0602
JM	12.3.73		P, A	0801-11, 0301	0201/2, 0401/2
AP	12.1.59			0401, 1301/16	0603, 0301/4
LR	16.3.63		P	0301, 0801-11	0201/2, 0401/2
ST	13.8.66		H, P	0103, 0301	0201/2, 0501-4
AW	28.12.35			0701, 0301	0201/2
DW	4.12.65	R	H, P, A	1302, 0301	0201/2, 0604-9
Healthy individuals					
NA	21.9.64			0301, 1501	0201/2, 0602
PBA	10.1.46			1302, 1501	0501-4, 0603/14
MD	25.10.71			1501, 0701	02, 0602
AG	21.10.51			1501, 0401-22	0602, 0301/4
CG	1.11.56			0102/4, 0701/3/4	0501-4, 0303
GH	2.10.46			1102/3, 1301	0603/14, 0301/4
JG	22.9.63			0301, 1501	0201/2, 0602
SH	17.12.68			1302, 1201-3	0604-9, 0301/4
JL	29.1.72			0101/5, 1201-5	0301/4, 0501-4
ML	26.10.40			0102, 0701	0201/2, 0501-4
BM	1.3.56			0301, 1401/7	0201/2, 0501-4
CR	24.4.71			*0103	*05
DS	3.3.64			0301, 1301	0201/2, 0603/14
HS	25.11.69			04, 0701	0201/2, 0302/7/8
JS	23.2.48			0801-11, 1301	04, 0603
PW*	15.1.61			11, 1302	0604, 0301

Table continued overleaf

Table 5.1

Subjects (* denotes male) enrolled into the study with the date of birth (D.O.B.), clinical symptoms, medication and HLA DR and HLA DQ type for each subject. Four groups were established:

- APS + β_2GP1 - SLE / APS patients with serum antibodies to β_2GP1
- APS - SLE / APS patients without serum antibodies to β_2GP1
- SLE - SLE patients
- healthy individuals

Symptoms:	A	arterial vascular disease
	C	cerebrovascular accident
	D	deep vein thrombosis
	E	eclampsia
	H	heart valve disease
	I	infertility
	L	livedo reticularis
	M	recurrent miscarriage
	P	pulmonary embolus
	R	renal disease
	S	ischaemic heart disease
	T	thrombocytopenia

Medication:	A	azathiopine
	C	cyclosporin
	H	hydroxychloroquine
	M	methotrexane
	P	prednisolone

β2GP1 antibodies (Table 5.2).

The HLA DR and HLA DQ haplotype of all the subjects was determined (Table 5.1) using PCR (Bunce *et al.*, 1995; Mullingham *et al.*, 1997).

5.2 Serum Antibody titre

5.2.1 Lupus anticoagulant and anticardiolipin antibodies

LAC data were obtained through clinical assessment. ACL titres were measured in serum collected on the day the T cell assay was performed using a commercial ELISA kit (see section 3.2). Normal serum has a titre of ≤ 11 Units/ml so values above this were considered positive. Of the 32 patients with clinical symptoms of APS (APS + β2GP1 and APS groups), 16 had LAC and ACL, 2 had LAC only, 8 had ACL only and 3 had neither LAC nor ACL but clear symptoms (Table 5.2). Three of the 32 patients were not tested for LAC since they were prescribed warfarin which interferes with the detection of LAC. Only one of these 3 patients had ACL. All 24 'SLE' patients were negative for serum LAC. Four of the 24 'SLE' patients had weak titres of ACL.

Serum ACL's were tested by 3 further independent sources; routine clinical analysis (QE Hospital, Birmingham), Dr. K.A. Davies at Imperial College, London and Dr. D. Wagenknecht, Methodist Hospital, Indianapolis. All methods assayed for IgG antibodies. Results were comparable in three of the four methods for all sera. The anomalies were due to some of the sera tested in the different ELISA's being collected on different dates or borderline titres in one assay coming up weakly positive or negative in another. Interestingly, of the 17 'APS' patients 10 had ACL of which 8 had recurrent miscarriages (Table 5.1 and 5.2). Of the 7 'APS' patients without ACL only one had recurrent miscarriages.

Table 5.2

	Lupus	Anticardiolipin	ß2GP1 IgG	
	anticoagulant	antibody	assay	historical
APS + ß2GP1				
KA	+	29.6	17.7	23.5
ACS	+	>100	52.2	>100
NH	+	>100	30.4	51.6
RHU	+	75.7	>100	>100
SH	+	83.2	>100	>100
AJ	+	29.5	84	80.3 §
LJ	+	>100	>100	>100
PM	+	17.1	28.5	37.6
MN	-	>100	>100	>100
GP	-	39.5	70.3	54.9
JPI	+	>100	37.9	58.2
MR*	+	>100	>100	>100
SS	-	>100	>100	>100
TW	ND (W)	>100	42.9	44.7
LY	+	>100	>100	>100
APS				
AB	-	20.3	3.3	5.4
KB	-	2.3	3.8	0.6
HCO	ND (W)	5.3	1.8	2.4
JF	+	20.3	8.5	7.6
RK	ND (W)	6.9	6.5	7.3
KL	+	20.7	12.5	10.2
MH	-	30.2	2.9	6.3
CO	+	3.1	3	3.2
JOR	+	61.1	7	16
DP	-	2.7	1.2	7
APO	-	12.9	4	7.5
RS	+	16.7	4.3	12.3 §
SUS	-	13.6	3.2	7.4
AT	-	16.3	2.9	5.3
LV	+	2.8	4.1	6.6
JW	-	2.9	0.7	0.4
NW	+	>100	5.4	9.6

Table continued overleaf

	Lupus	Anticardiolipin	ß2GPI IgG	
	anticoagulant	antibody	assay	historical
SLE				
JA	-	8.3	0.9	3.1
SA	-	9.6	0.1	1.9
CB	-	11.1	1.3	3.1 #
JB	-	1.9	1.9	1.8
MB	-	8.8	0.3	1.1
MBO*	-	9.8	2.3	4.2
AD	-	4.1	1.7	3 #
KD	-	16.1	2.4	2.8
MDR	-	11.5	13.4	7.5 #
AF	-	3.8	2.6	1.4 #
CF	-	3.9	1.4	5.8
BGR	-	16.1	12.7	14.6
KH	-	6.4	2.4	1.8
RH	-	10.7	1.5	2.7
HJ	-	4.9	1.7	3.1
PK	-	4.7	8.1	4.6
HM	-	20.1	10.2	4.1
HMO	-	7.8	2.8	1.5
JM	-	6.9	4.5	4.5
AP	-	8.4	2.8	1.2
LR	-	11.2	2.7	3 #
ST	-	3.7	3	3.8
AW	-	12.8	2.1	4.5
DW	-	3.1	10.4	7.4 #
Healthy individuals				
NA		6.1		6.8
PBA		2.3		8.9
MD		7.8		1.9
AG		1.3		3.5
CG		2.2		1.3
GH		6.4		0
JG		3		7.3
SH		7.9		4.6
JL		2.4		7.5
ML		3.9		1.2
BM		3.8		2
CR		5.9		10.6
DS		2.6		1.3
HS		14.9		1.8
JS		3.9		2.6
PW*		3.9		2.1

Table continued overleaf

Table 5.2

Subjects (* denotes male) enrolled into the study listing the presence of lupus anticoagulant, anticardiolipin antibodies and anti - β2GP1 antibodies in the serum of each subject. Lupus anticoagulant data was obtained through clinical assessment, while anticardiolipin antibodies and anti - β2GP1 antibodies were determined by several methods (see section 3.2). The table reports the results obtained from an anticardiolipin antibody ELISA kit supplied from The Binding Site and an anti - β2GP1 antibody ELISA kit supplied by Shield Diagnostics. Anti - β2GP1 antibody titres were determined in serum samples taken at the time of the proliferation assay and at 6 months to 3 years prior to the proliferation assay. Results obtained by the different methods were comparable except for the following:

§ denotes negative by Imperial College School of Medicine (Dr. K.A.Davies)

# denotes positive by Imperial College School of Medicine (Dr. K.A.Davies)

5.2.2 Anti - β2 glycoprotein 1 Antibodies

Recently published data suggest that serum anti - β2GP1 antibodies are a better diagnostic marker for APS than LAC or ACL (Cabiedes *et al.*, 1995; Guerin *et al.*, 1997). Due to the debate over how anti - β2GP1 antibodies should be measured and their relevance (Cantini *et al.*, 1998; Inanc *et al.*, 1997) in this present study serum IgG anti - β2GP1 antibodies were measured by three different ELISA's. Antibody titres were measured using either an ELISA kit supplied by Shield Diagnostics or Genesis, and by the Imperial College School of Medicine, London (Dr. M. Samarkos and Dr. K.A. Davies). The results obtained from the ELISA kit supplied by Shield Diagnostics are given in Table 5.2. Titres above 10 Units/ml are considered positive. Sera collected at two time points were tested using this kit: serum collected on the day the T cell assay was performed (referred to as 'assay') and serum collected during the previous 6 months to 3 years and stored at -80°C (referred to as 'historical'). All samples were tested using the ELISA kit from Shield Diagnostics. 'Assay' samples were confirmed using the ELISA kit from Genesis while 'historical' samples were confirmed by the Imperial College School of Medicine, London.

All 'assay' samples gave identical results, in terms of the magnitude of the antibody titre, on the Shield Diagnostics and the Genesis ELISA kits. However, for 8 'historical' samples titres differed between the Shield Diagnostics kit and data obtained from the Imperial College School of Medicine (see section 5.2). Six of these 8 samples were weakly positive when tested at the Imperial College but negative when tested using the Shield Diagnostic kit. Two of these six patients had borderline titres on 'assay' samples. 'Assay' serum samples from all six patients were therefore tested at the Imperial College and found to be negative. For all of the 56 patients anti - β2GP1 antibody titres did not vary significantly over time.

Ten of the 15 'APS + β2GP1' patients, all with a high serum anti - β2GP1 antibody titres, had the HLA DR4 haplotype. To confirm that the ELISA was specific for anti - β2GP1 antibodies and not HLA DR4 serum from ten patients with Felty's syndrome was tested. All ten Felty's syndrome patients had the HLA DR4 haplotype. However, serum from all

of the patients was negative in the ELISA. Serum from the healthy individuals was tested using the Shield Diagnostic ELISA kit only and all were found to be negative for anti - β2GP1 antibodies (Table 5.2).

To test the hypothesis that β2GP1 is associated with apoptosis antibodies to phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine were tested in 36 of the 56 serum samples. Antibodies to PS, PC and PE were also tested for their dependence on cofactors, particularly β2GP1, for their binding. This work was performed by Dr. D. Wagenknecht at the Methodist Hospital of Indiapolis (Goldberg *et al.*, 1996). The ELISA was performed in BSA to determine which antibodies bind independently of cofactors and in bovine serum to determine antibodies which are dependent on cofactors for binding. Samples positive in the ELISA containing bovine serum were tested specifically for dependence on β2GP1 for binding. Of the 38 patients none had IgG antibodies to PE and 4 had IgG antibodies to PC which were independent of cofactors for binding to the phospholipid. Thirteen of the 38 samples were positive for antibodies to PS of which 12 were dependent on β2GP1 for binding (Table 5.3). All of the 56 patients were tested for ACL of which 28 were positive (Table 5.3). Eighteen of the positive ACL samples were dependent specifically on β2GP1 for binding and one was dependent on a cofactor but not β2GP1. Of the remaining 9 patients 7 had ACL which were independent of cofactor for binding while two were not tested for the requirement of a cofactor. Interestingly, these 7 patients did not have any symptoms of APS.

5.3 HLA haplotypes

HLA frequencies were examined in all of the 16 healthy individuals and the 56 patients enrolled into the study. The HLA haplotypes of the individuals are shown in Table 5.1. The HLA frequencies for the HLA haplotypes associated with APA's and anti - β2GP1 antibodies are shown in Table 5.4. The most striking difference in HLA haplotype data was seen in the 'APS + β2GP1' patients in which the occurrence of the HLA DR4 was increased (uncorrected $p = 0.0026$, corrected $p = 0.026$). HLA DQβ1*0302 was increased

Table 5.3

	PS antibodies			ACL antibodies		
	Albumin	**Serum**	**ß2GP1**	**Albumin**	**Serum**	**ß2GP1**
APS + ß2GP1 Ab						
KA	-	-		-	+	-
ACS	+	+	+	+	+	+
NH	+	+	+	+	+	+
RHU	+	+	+	+	+	+
SH	+	+	-	+	+	+
AJ	-	-		-	-	
LJ	+	+	+	+	+	+
PM	+	+	+	+	+	+
MN	ND			+	+	+
GP	+	+	+	+	+	+
JPI	+	+	+	+	+	+
MR*	ND			+	+	+
SS	ND			+	+	+
TW	ND			+	+	+
LY	+	+	+	+	+	+
APS						
AB	-	-		-	-	
KB	-	-		-	-	
HCO	-	-		-	-	
JF	±	ND	+	+	+	+
RK	-	-		-	-	
KL	-	-		-	-	
MH	+	+	+	+	+	+
CO	-	-		-	-	
JOR	+	+	+	+	+	+
DP	-	-		-	-	
APO	-	-		+	-	
RS	+	+	ND	+	+	+
SUS	-	-		+	ND	ND
AT	-	-		-	-	
LV	ND			-	-	
JW	-	-		-	-	
NW	ND			-	-	

Table continued overleaf

Table 5.3 continued

	PS antibodies			ACL antibodies		
	Albumin	**Serum**	**ß2GP1**	**Albumin**	**Serum**	**ß2GP1**
SLE						
JA	ND			-		
SA	-	-		-	-	
CB	-	-		-	-	
JB	ND			-	-	
MB	-	-		-	-	
MBO*	-	-		-	-	
AD	-	-		-	-	
KD	ND			+	-	-
MDR	-	-		-	-	
AF	-	-		-	-	
CF	-	-		-	-	
BGR	+	-	+	+	-	+
KH	ND			+	-	-
RH	ND			+	-	-
HJ	ND			+	-	-
PK	ND			+	-	-
HM	ND			+	-	-
HMO	ND			-	-	
JM	ND			-	-	
AP	-	-		-	-	
LR	-	-		-	-	
ST	ND			-	-	
AW	ND			+	-	-
DW	-	-		-	-	

Table 5.3

Subjects (* denotes male) enrolled into the study listing the presence of serum anti - phosphatidylserine and anticardiolipin antibodies in each of the patients. To determine whether the antibody was independent, or dependent on cofactors or β_2GP1, for binding antibody titres were measured in the presence of bovine serum albumin, bovine serum or human β_2GP1 respectively (± borderline antibody titre; + positive titre; - negative titre; ND not determined). Only samples positive in bovine serum were tested for dependence on β_2GP1 for binding. Data was supplied by Methodist Hospital of Indiapolis (Dr. D. Wagenknecht).

HLA haplotype	APS + ß2GP1	APS	SLE	Controls
	N° (%)	N° (%)	N° (%)	N° (%)
DR2	2 (13)	2 (12)	9 (38)	5 (31)
DR4	10 (67)	3 (18)	3 (13)	2 (13)
DR6	5 (33)	3 (18)	5 (21)	6 (38)
DR7	2 (13)	4 (24)	3 (13)	4 (25)
DQ2	7 (47)	14 (82)	15 (63)	7 (44)
DQ3	10 (67)	5 (29)	6 (25)	6 (38)
DQß1*0301/4	5 (33)	5 (29)	4 (17)	4 (25)
DQß1*0302	6 (40)	1 (6)	0 (0)	0 (0)
DQ6	7 (47)	4 (24)	14 (58)	10 (63)
DQß1*0604-9	2 (13)	2 (12)	2 (8)	0 (0)
n =	15	17	24	16

Table 5.4

HLA haplotype frequencies in patients with SLE, APS and anti - β_2GP1 antibodies ('APS + β_2GP1'), SLE and APS without anti - β_2GP1 antibodies ('APS'), patients with SLE ('SLE') and healthy individuals (controls). HLA DR2 subtypes are represented by HLA DRβ1*15. HLA DR6 subtypes are represented by HLA DRβ1*13. Only HLA haplotypes associated with the presence of serum anti - β_2GP1 antibodies were examined.

in 'APS + β2GP1' patients but corrected P values did not reach a significant difference (uncorrected p = 0.007, corrected p = 0.098). HLA DQ6 was reduced in 'APS' patients but corrected P values did not reach a significant difference (uncorrected p = 0.023, corrected p = 0.115).

5.4 T cell proliferation

The protein β2GP1 is hypothesised to be a potential autoantigen in SLE and / or APS since it is required for the binding of ACL to cardiolipin (Alarcon-Segovia *et al.*, 1997; Roubey *et al.*, 1995; Tsutsumi *et al.*, 1996). The aim of this section of work was therefore to determine whether T cells specific to β2GP1 could be measured in the peripheral blood of patients with ACL or anti - β2GP1 antibodies. The 7 day assay system developed for responses to recall and naive antigen was used (see section 4.1). Individual values for thymidine incorporation following stimulation with antigens are given in Appendix A.

5.4.1 Optimum concentration of β2 glycoprotein 1

Figure 5.1 shows the proliferative responses of PBMC's from a healthy individual (ML) and two patients with 'APS + β2GP1' (LJ and ACS) to β2GP1. The β2GP1 was supplied purified by Serbio. The optimum response to β2GP1 occurred at a final concentration of 10μg/ml. This concentration was used to examine T cell responses to β2GP1 in the remaining subjects.

5.4.2 Proliferative response to β2 glycoprotein 1

Proliferative responses to standard antigens and β2GP1 was determined in the 16 healthy individuals and 56 patients. CPM values for each subject are given in Appendix A. The data are summarised in Table 5.5 and Table 5.6. Triplicate wells were established for tetanus toxoid and PPD while between 5 and 14 replicate wells were established for β2GP1 and rabies protein. The number of replicate wells varied between subjects and depended on

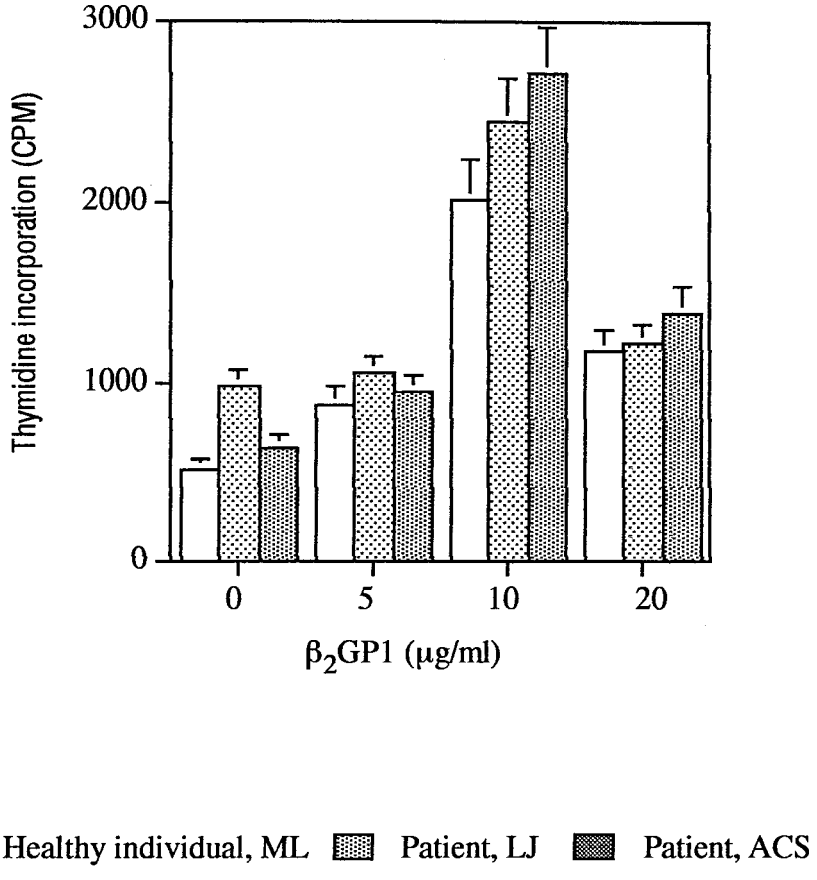


Figure 5.1

Proliferative responses (tritiated thymidine incorporation) obtained from PBMC's taken from a healthy individual and two patients with SLE and APS with anti - β2GP1 antibodies ('APS + β2GP1'). Triplicate cultures of $2x10^5$ cells per well were incubated for 7 days with β2GP1 at 5μg/ml, 10μg/ml and 20μg/ml. Anti - CD28 antibody was added to aid costimulation. The results are expressed as mean CPM ± standard deviation.

Table 5.5

	ß2GP1	Rabies	TT	PPD	HLA DR	HLA DQ
APS + ß2GP1						
KA			+	+	0101, 0401-22	05, 0301/4
ACS	+		+	+	1301, 0401	0603, 0301/4
NH				+	0304, 1101/4/6	02, 0301
RHU				+	0701, 1301	0201/2, 0603
SH			+	+	0401/4	0301/4, 0302
AJ			+	+	0404, 0301	0201/2, 0302
LJ			+	+	0101/2/4, 0401	05, 0302
PM			+	+	0401, 0304	02, 0302
MN	+	+	+	+	0301, 1302	0604-9, 0201/2
GP					1001, 1302	05, 0604-9
JPI				+	1501, 0401-22	0602, 0302
MR*		+		+	0301, 0401-22	0301/4, 0201/2
SS			+	+	0401-22	*0302
TW	+	+	+	+	1501, 1301	0601, 0603
LY	+			+	0401, 0701	02, 0601/2
APS						
AB				+	1501, 0301	0201/2, 0602
KB			+	+	*0301	*0201/2
HCO					0101, 0301	0201/2, 0501-4
JF				+	0401, 1302	0604-9, 0301/4
RK				+	0401, 0301	0201/2, 0301/4
KL				+	0402, 13	0301/4, 0302
MH				+	0701, 1401/7	0201/2, 0501-4
CO	+		+	+	0301, 0701	*02
JOR		+	+	+	1501, 1401/7	0501-4, 0601/2
DP				+	11, 0301	0201/2, 0301/4
APO	+	+	+	+	0301, 1302	0201/2, 0604-9
RS					0103, 0901	0201/2, 0501-4
SUS		+	+		*0301	0201/2
AT			+	+	0301, 0701	0201/2
LV	+	+	+	+	0301, 11	0201/2, 0301/4
JW	+		+	+	*0304	*02
NW			+	+	0301, 0701	0201/2

Table continued overleaf

Table 5.5 continued

	ß2GP1	Rabies	TT	PPD	HLA DR	HLA DQ
SLE						
JA				+	1301, 1303	0603, 0301/4
SA				+	1601-6, 0301	0201/2, 0501-4
CB	+			+	1501, 0401	0602, 0301/4
JB			+	+	1501-5, 0301	0601/2, 02
MB	+		+	+	1501, 0301	0201/2, 0602
MBO*	+	+		+	0701, 1302	0201/2, 0604-9
AD				+	1501-5, 0901	*06, 09
KD	+		+	+	0101, 0401	0501-4, 0301/4
MDR		+	+	+	0101, 0301	0201/2, 0501-4
AF			+		1501-5, 0801-11	04, 0602
CF			+	+	1501	*0602
BGR			+	+	0101, 0301	0201/2, 0501-4
KH			+		0701, 1501	0303, 0602
RHO			+	+	*0901, 0304	02, 0303
HJ	+	+	+	+	1501, 0301	0201/2, 0602
PK	+		+	+	1301, 0301	0201/2, 0603
HM					*0301	*02, 02
HMO	+	+		+	1501	*0602
JM					0801-11, 0301	0201/2, 0401/2
AP			+		0401, 1301/16	0603, 0301/4
LR				+	0301, 0801-11	0201/2, 0401/2
ST			+	+	0103, 0301	0201/2, 0501-4
AW		+	+	+	0701, 0301	0201/2
DW			+		1302, 0301	0201/2, 0604-9
Healthy individuals						
NA					0301, 1501	0201/2, 0602
PBA		+	+	+	1302, 1501	0501-4, 0603/14
MD		+	+	+	1501, 0701	02, 0602
AG		+	+	+	1501, 0401-22	0602, 0301/4
CG		+	+	+	0102/4, 0701/3/4	0501-4, 0303
GH		+	+	+	1102/3, 1301	0603/14, 0301/4
JG		+	+	+	0301, 1501	0201/2, 0602
SH			+	+	1302, 1201-3	0604-9, 0301/4
JL		+	+	+	0101/5, 1201-5	0301/4, 0501-4
ML	+	+	+	+	0102, 0701	0201/2, 0501-4
BM		+	+	+	0301, 1401/7	0201/2, 0501-4
CR			+	+	*0103	*05
DS		+		+	0301, 1301	0201/2, 0603/14
HS			+	+	04, 0701	0201/2, 0302/7/8
JS				+	0801-11, 1301	04, 0603
PW*			+	+	11, 1301	0604, 0301

Table continued overleaf

Table 5.5

Summary of mean proliferative responses to β_2GP1, rabies protein, tetanus toxoid (TT) and PPD in patients with SLE and APS with anti - β_2GP11 antibodies ('APS + β_2GP1'), patients with SLE and APS without anti - β_2GP1 antibodies ('APS'), patients with SLE ('SLE') and healthy individuals. The HLA haplotype of each subject is given. A full set of CPM values are given in Appendix A.

\+ denotes subjects who respond to the antigen with a SI $\geq$ 3.

Antigen	Healthy individuals	APS + ß2GP1	APS	SLE
Medium	453 ± 260	581 ± 278	548 ± 444	487 ± 378
TT	29036 ± 32448	9486 ± 11233	7382 ± 11135	7505 ± 9780
PPD	41469 ± 39969	13465 ± 12397	14214 ± 23222	6888 ± 9395
Rabies	1624 ± 1541	1094 ± 657	954 ± 875	1218 ± 1839
ß2GP1	683 ± 500	1477 ± 1033	1062 ± 1183	1792 ± 2551

Table 5.6

Mean proliferative responses to medium, tetanus toxoid (TT), PPD, rabies protein and β_2GP1 in healthy individuals, and 'APS + β_2GP1', 'APS' and 'SLE' patients. The results are expressed as mean CPM for the group of subjects ± standard deviation.

the number of PBMC's obtained. The mean proliferative responses to tetanus toxoid and PPD were higher in healthy individuals compared to patients. There was no significant difference in the number of subjects in each group responding to PPD. However, proportionally fewer patients responded to tetanus toxoid compared to healthy individuals. The mean proliferative response to β2GP1 and rabies protein was similar in healthy individuals and patients (Table 5.6). However, only one of the 16 healthy individuals but 15 of the 56 patients showed a positive response to β2GP1. Ten of the 16 healthy individuals and 12 of the 56 patients showed a positive proliferative response to rabies protein. The proliferative response to β2GP1 was not restricted by the clinical diagnosis nor by the presence of serum antibodies to β2GP1, LAC or ACL. The response to β2GP1 did not reflect the ability to respond to rabies protein, tetanus toxoid or PPD. Neither was there any clear association between the ability to proliferate in response to stimulation in vitro with β2GP1 and the HLA haplotype (Table 5.7).

5.4.3 Alternative source of β2 glycoprotein 1

To confirm that the proliferative response to β2GP1 was not due to a poor antigen preparation or contamination an additional source of β2GP1 was tested. This antigen was supplied purified by Immunodiagnostic Research Laboratories (IDRL). Purity was verified on a SDS PAGE gel (Figure 5.2). Proliferative responses to β2GP1 supplied by Serbio and IDRL were determined in two patients (LV and LY) and one healthy individual (ML). These subjects showed a good proliferative response to β2GP1 obtained from Serbio (Table 5.5). The proliferative responses to both antigen preparations were very similar for all three subjects (Figure 5.3).

5.4.4 Exposure to β2 glycoprotein 1

The proliferative response to tetanus toxoid, PPD, rabies protein and β2GP1 was measured over a 14 month period in one healthy individual (MD). The results are shown in Figure 5.4. The individual had been vaccinated with tetanus toxoid within the last ten years and

HLA	APS + ß2GP1		APS		SLE		Controls	
	Total N° subjects	ß2GP1 response	Total N° subjects	ß2GP1 response	Total N° subjects	ß2GP1 response	Total N° subjects	ß2GP1 response
DR2	2	1	1	0	9	5	5	0
DR4	9	2	3	0	3	2	2	0
DR6	6	3	4	1	4	1	6	0
DR7	2	1	4	1	2	1	4	1
DQ2	6	2	14	4	15	4	7	1
DQ3	10	1	5	1	6	2	6	0
DQ6	7	4	4	1	14	6	10	0

Table 5.7

The number of subjects with a particular HLA haplotpye in each of the subject groups and the number of those subjects who responded to β_2GP1 *in vitro*. HLA DR2 subtypes are represented by HLA DRβ1*15. HLA DR6 subtypes are represented by HLA DRβ1*13. Only HLA haplotypes associated with the presence of serum anti - β_2GP1 antibodies were examined.

'APS + β_2GP1' denotes SLE, APS and anti - β_2GP1 antibodies

'APS' denotes SLE and APS without anti - β_2GP1 antibodies

'SLE' denotes patients with SLE

Controls denotes healthy individuals

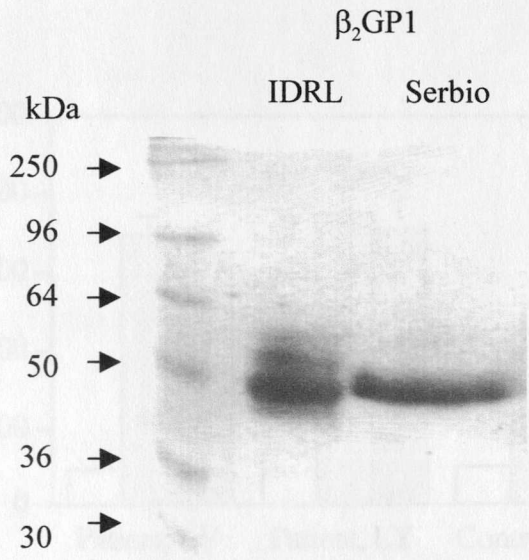


Figure 5.2
SDS PAGE gel of β_2 glycoprotein 1 obtained from IDRL and Serbio (see section 3.4.3). Proteins were loaded at 10μg per lane.

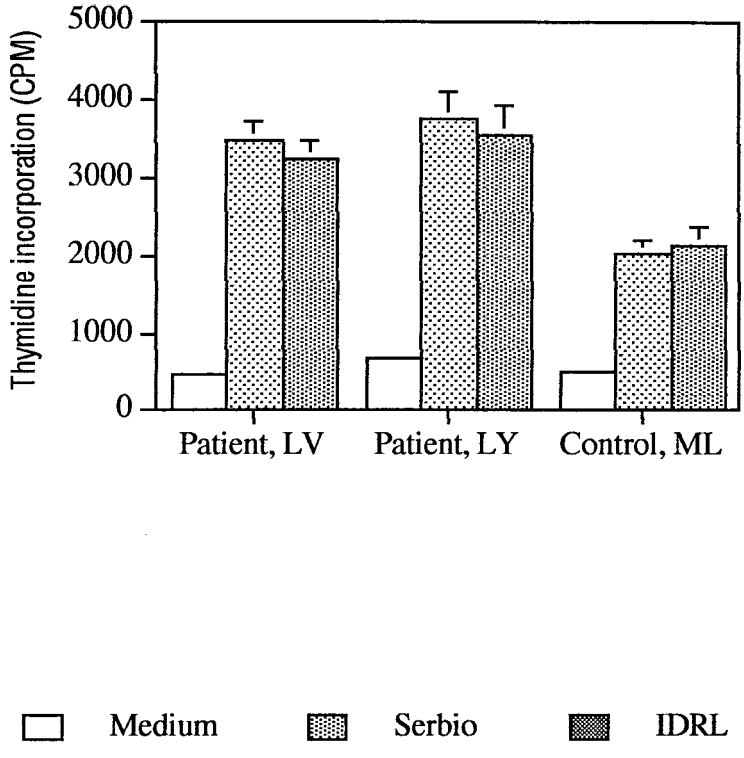


Figure 5.3

Comparable proliferative response (tritiated thymidine incorporation) in two patients (LV and LY) and a healthy individual (ML) to two different sources of β2GP1 (10μg/ml). β2GP1 obtained from Serbio was used throughout the study and compared with β2GP1 obtained from IDRL in these three subjects. Proliferation assays were carried out as described in section 3.3.9. The results are expressed for as mean CPM of the replicates ± standard deviation.

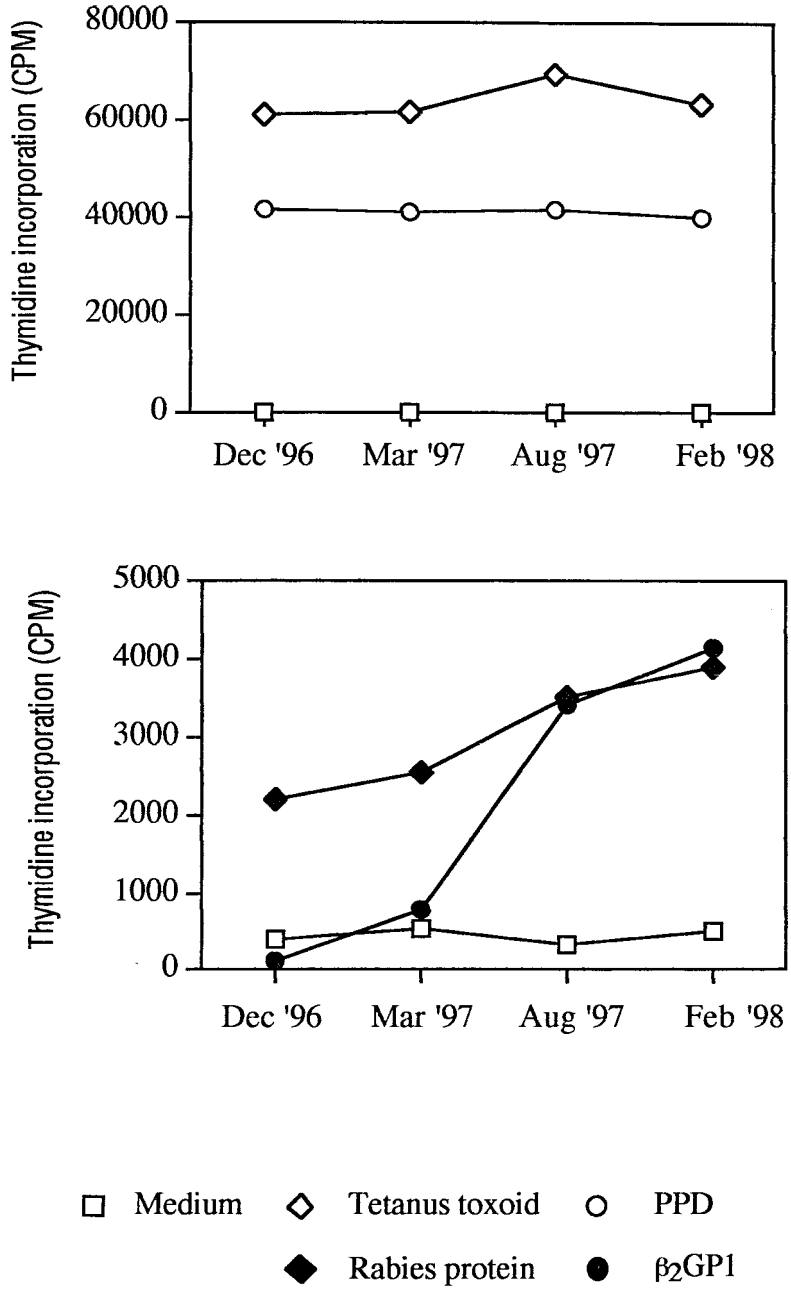


Figure 5.4

Proliferative responses (tritiated thymidine incorporation) to stimuli; tetanus toxoid, PPD, rabies protein and β_2GP1 over a 14 month exposure in a healthy individual (MD).

showed a good proliferative response *in vitro*. This proliferative response to tetanus toxoid, PPD and medium did not vary throughout the time period. Prior to exposure to β_2GP1, the individual did not show a proliferative response to the antigen. However, continuous exposure during experiments over the 14 months resulted in an increased proliferative response with the greatest increase occurring between March 1997 and August 1997. However, the individual remained seronegative for anti - β_2GP1 antibodies and developed no clinical features of APS or SLE. The response to rabies protein also increased over time with continuous exposure although the individual had been exposed to the antigen prior to December 1996.

5.5 Discussion

5.5.1 β_2 glycoprotein 1 as a T cell autoantigen

This study confirms published reports of a striking association between the presence of serum anti - phospholipid antibodies, antibodies to phospholipid cofactors and clinical manifestations of APS (Cabiedes *et al.*, 1995; Alarcon-Segovia *et al.*, 1997; Guerin *et al.*, 1997; Tsutsumi *et al.*, 1996). However, the role of autoantibodies in the pathogenesis of the disease and the nature of the target autoantigen(s) remains elusive. B cell epitope studies have revealed anti - phospholipid antibody activity requires the cofactor β_2GP1 for binding (Hunt *et al.*, 1993; Iverson *et al.*, 1998; George *et al.*, 1998). This suggests that this cofactor is recognised by T cells in the immune system and acts as the target autoantigen. Robbins *et al.* (1998) report that β_2GP1 complexed to cardiolipin increases platelet aggregation, and activation and hence provides a link with thrombosis. Blank *et al.* (1995) showed anti - β_2GP1 antibodies, but not ACL antibodies, developed in mice receiving bone marrow taken from a mouse with experimental APS. T cell depleted bone marrow was unable to transfer the disease. This indicated that the antibody response was dependent on T cells. Several groups report that immunisation of rabbits and mice with human β_2GP1 leads to the induction of antibodies to the protein as well as to other negatively charged phospholipids, including cardiolipin (Kouts *et al.*, 1995; Pierangeli and

Harris, 1993; Gharavi *et al.*, 1992; Rauch and Janoff, 1992). However, ACL can only be generated by immunisation with cardiolipin plus β_2GP1 and not with cardiolipin alone (Rauch and Janoff, 1992). Despite the huge efforts to elucidate the immunological mechanism(s) involved in the production of anti - phospholipid antibodies much remains unsolved. However, the interaction of β_2GP1 with the phospholipid postulates β_2GP1 is a T cell target autoantigen in APS/SLE.

5.5.2 HLA associations with anti - β_2GP1 antibodies and β_2GP1 specific T cells

Antigen is presented to T cells in the context of the MHC molecule. Disease associated HLA DR and HLA DQ haplotypes may therefore be suggestive of a disease mechanism. In the present study an attempt was not made to uncover HLA associations with disease or the presence of serum antibodies. These data are already published; HLA DR4 and HLA DQβ1*0302 are increased while HLA DR2 and DQ6 are reduced in patients with APS (Goldstein *et al.*, 1996; Arnett *et al.*, 1999; Asherson *et al.*, 1992). However, the data obtained in the present study are consistent with these published reports although P values did not reach statistically significant levels in some instances probably because too few patients were studied.

Many of the HLA haplotypes associated with SLE and APS are in strong linkage disequilibrium with MHC and non MHC related genes. Hence, it is possible that the reported HLA disease associated haplotypes do not play a role in the pathogenesis of the disease. More than 90% of patients with ankylosing spondylitis are HLA B27 positive. Although the HLA B27 association is considered helpful in the diagnosis of ankylosing spondylitis it is also possible that subjects without this haplotype may also develop the disease. Additionally, there is insufficient knowledge regarding the relationship between haplotypes and the interaction of MHC molecules with the TCR and the T cell response. Patients developing myasthenia gravis before the age of 40 years show an association with HLA DR3 and HLA B8 (these HLA types are in linkage disequilibrium). However, the disease is dependent on Th cells but has a stronger association with HLA B8 than HLA DR3 (Hawke *et al.*, 1996).

5.5.3 T cell proliferative response to β_2 glycoprotein 1

To address the hypothesis that β_2GP1 is a T cell target autoantigen in SLE and / or APS PBMC's from patients with SLE and / or APS were cultured with β_2GP1 for 7 days. Proliferation was measured by thymidine incorporation during the final 18 hours of culture (see section 3.3.9). A proliferative response was considered positive if the thymidine incorporation was greater than three times the proliferative response to medium. Proliferation was observed in 33% of patients with antibodies to cardiolipin and β_2GP1 who had clinical features of APS ('APS + β_2GP1'). Visvanathan and McNeil (1999) report T cell responses to β_2GP1 in 44% of 'APS + β_2GP1' patients but if a SI of 3 is used as the cut off point for a positive response only 28% of the patients responded to β_2GP1. Additionally, in the present study, 24% of patients with clear symptoms but no antibodies typical of APS ('APS'), 29% of patients diagnosed with SLE ('SLE') and one of 16 (6%) healthy individuals responded to β_2GP1 (Table 5.5). Visvanathan and McNeil (1999) did not study 'APS' patients but showed that PBMC's from patients with ACL and anti - β_2GP1 antibodies, but without the clinical features of APS, did not proliferate in response to β_2GP1. Importantly, in contrast to this thesis, Visvanathan and McNeil (1999) did not identify proliferative responses to β_2GP1 in 7 patients with SLE or arthritis. The difference may be due to the number of patients studied and their individual symptoms.

This present study and the study of Visvanathan and McNeil (1999) show that a T cell response to β_2GP1 is not associated with the presence of ACL or anti - β_2GP1 antibodies. The relevance of β_2GP1 specific T cells to disease is unclear since the two studies report opposing data. However, anti - β_2GP1 antibodies remain a good clinical marker for APS.

5.5.4 The assay system

The assay system used in the present study was supplemented with human serum and therefore also contains β_2GP1 (Polz *et al.*, 1979). However, very low levels of proliferation

were observed to medium alone and a titration of exogenous β_2GP1 showed a dose response with optimum proliferation at 10μg/ml (Figure 5.1). To overcome any problems associated with the presence of β_2GP1 in the serum, a serum free assay should have been used. Visvanathan and McNeil (1999) report T cell responses to β_2GP1 added to a serum free assay. However, the proliferative response to the serum free medium alone was high (900 CPM). Serum free media have been developed specifically for cultivating hybridoma cell lines. Hence, a serum free assay was not considered as ideal for the present study using PBMC's. For this reason medium supplemented with human serum was selected for these studies.

Visvanathan and McNeil (1999) report proliferative responses to 1% normal serum added to serum free medium in patients responding to β_2GP1. The proliferative response to normal serum was greater than the proliferative response to β_2GP1 (25μg/ml) despite 1% normal serum containing only 2μg/ml β_2GP1. The patients did not respond to normal serum depleted of β_2GP1 but a depletion with a control antibody stimulated a greater proliferative response than non - depleted serum. Patients who did not respond to β_2GP1 did not respond to normal serum. It was suggested that β_2GP1 present in serum was responsible for the proliferative response. In the present study patients who responded to β_2GP1 did not show an abnormally high proliferative response to the medium containing normal serum. However, some patients studied showed a background response of greater than 900 CPM (LJ, PM, KB, CO, JOR and MDR). There was no association between an increased response to medium in these six patients and the ability to respond to β_2GP1, serum antibody titres or disease. However, it can not be excluded that β_2GP1 present in normal serum was involved in the proliferative response.

5.5.5 Continuous exposure to β_2 glycoprotein 1

During the present study a healthy individual (MD) was exposed on a regular basis to β_2GP1 whilst carrying out experiments. Prior to exposure a proliferative response to β_2GP1

was not detected *in vitro*. However, following exposure to the protein over a 14 month period a proliferative response was detected *in vitro* suggesting that the autoantigen specific T cells are not deleted during positive and negative selection in the thymus. This suggests that β_2GP1 is not present in the thymus (although it is a serum protein and binds apoptotic cells), that the threshold level for detection is not reached or that β_2GP1 epitopes are not presented during T cell development. This is supported by the finding that β_2GP1 must be at a high density and undergo conformational changes before it is functional (Borchman *et al.*, 1995; Matsuura *et al.*, 1994).

The healthy individual (MD) did not develop antibodies to cardiolipin or β_2GP1 nor develop clinical manifestations of the disease. This reinforces the conclusion that β_2GP1 specific T cells are not associated with anti - β_2GP1 antibodies. It is hypothesised that a low level T cell response has been generated but that the response is insufficient in quantity or quality to lead to antibody production.

The individual had the HLA haplotype DR7, 15; DQ2, 6; a haplotype not normally associated with the development of APS or APA's. Arnett *et al.* (1999) report no difference in the frequency of HLA DR7 in patients compared to healthy individuals. However, Savi *et al.* (1998) and Granados *et al.* (1997) report increased occurrence of HLA DR7 in patients with SLE from Southern Europe and Mexico. Additionally, Vargas-Alarcon *et al.* (1997) report an increase in HLA DR7 in Caucasian patients taking chlorpromazine who do not have SLE but have lupus anticoagulant. Intriguingly, the only healthy individual (ML) to show a proliferative response to β_2GP1 *in vitro* has the HLA DR2, 7; DQ2, 5 haplotype (Table 5.5). This suggest that HLA DR7 may play a role in the presentation of β_2GP1 to T cells.

5.5.6 Naive or memory β_2 glycoprotein 1 specific T cells

An interesting point arising from the present study is the difference in the ability of subjects to respond to rabies protein compared to β_2GP1. Of the 'APS + β_2GP1' patients

13.3% responded to rabies protein but 33% responded to β_2GP1. In comparison, the response to rabies protein or β_2GP1 in 'APS' patients was 24% and 24%, respectively, and in 'SLE' patients the response was 21% and 29%, respectively. However, 59% of healthy individuals responded to rabies protein but only one of the 16 (6%) responded to β_2GP1. The data imply that although the T cell response to β_2GP1 is poor in patients β_2GP1 specific T cells play a significant role in the development of the disease. The present study does not attempt to determine the nature of the T cells mediating the proliferative response to β_2GP1. However, Papo *et al.* (1994) suggest that CD4 T cells from patients with APS have a defective activation pathway impeding the conversion of naive cells into memory cells. To support this, Byrne *et al.* (1988) report that naive and memory T cells have different triggering requirements. Hence, the proliferative responses to rabies protein and β_2GP1 in the present thesis may be explained by these predictions. It is therefore hypothesised that T cell responses to β_2GP1 are low level primed T cells which are unable to expand and differentiate into fully matured cells.

5.5.7 Conformational changes within β2 glycoprotein 1

This present study and Visvanathan and McNeil (1999) report that β_2GP1 is not a T cell target autoantigen involved in the production of anti - β_2GP1 antibodies. However, the molecular weight of β_2GP1 by Western blotting suggested that it was correctly glycosylated. It was therefore presumed that β_2GP1 was processed and presented *in vitro* in the normal manner. However, β_2GP1 has been shown to undergo conformational changes following its interaction with phospholipids and other negatively charged surfaces (Tsutsumi *et al.*, 1996; Arvieux *et al.*, 1991). This conformational change may yield neo - epitopes ('cryptic') which drive the immune response (Matsuura *et al.*, 1994; Borchman *et al.*, 1995; Roubey *et al.*, 1995). These epitopes may not have been presented in either of the proliferation assay systems used to identify β_2GP1 specific T cells. It therefore remains a possibility that β_2GP1 expressed in an alternative conformation is the T cell autoantigen. Alternatively, another cofactor such as prothrombin, protein C or protein S is the T cell

antigen or is acting as a 'hapten' carrier for β_2GP1 in the production of anti - β_2GP1 antibodies. It is suggested that inclusion of a β_2GP1 - phospholipid antigen or alternate cofactor are examined in future assay systems.

5.5.8 The role of apoptosis in the proliferative response to β_2 glycoprotein 1

Phospholipids, particularly phosphatidylserine which is normally found on intracellular membranes, are exposed during apoptosis and become accessible to the immune system (Fadok *et al.*, 1992; Savill *et al.*, 1993). Drouvalakis and Buchanan (1998) report that patients with serum lupus anticoagulant have increased antibody titres to the phospholipids phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine. In the present study none of the 38 patients tested had antibodies to PE and only four had antibodies to PC of which none were dependent on β_2GP1 for binding. However, 12 of 13 patients with serum antibodies to PS showed a dependency for β_2GP1 in the detection of the antibody (Table 5.3). This is in agreement with McNeil *et al.* (1990) showing that some APA's require β_2GP1 for binding to phospholipids. It is therefore hypothesised that apoptosis is important in SLE and / or APS.

The binding of anti - phospholipid antibodies to phospholipids, via β_2GP1, is enhanced when β_2GP1 is bound to a negative surface (Alarcon-Segovia *et al.*, 1997; Sheng *et al.*, 1998). This negative surface includes the surface of apoptotic cells. β_2GP1 binding to apoptotic cells facilitates the clearance of anti - phospholipid opsonised apoptotic cells (Manfredi *et al.*, 1998a). However, Herrmann *et al.* (1998) report that in SLE there is a failure to clear apoptotic cells. It is therefore proposed that β_2GP1 specific T cells interfere with the binding of anti - PS antibodies to the PS / β_2GP1 complex on the apoptotic cell surface through simple competition. This would therefore inhibit the removal of apoptotic cells by opsonisation, lead to the accumulation of apoptotic material and promote the availability of autoantigens to the immune system. Additionally, the binding of phospholipids to β_2GP1 may reveal cryptic epitopes which become accessible to T cells.

This may explain why a T cell response to β_2GP1, but not an antibody response, is associated with patients with APS and / or SLE.

Chapter 6

Results Section 3

La protein as a T cell autoantigen

High titres of autoantibodies directed against the La protein (48kDa) are found in patients with SLE and primary Sjogren's Syndrome (SS). (Tomer *et al.*, 1993). Mice immunised with La develop antibodies to La which spreads with time to include anti - Ro antibodies (Topfer *et al.*, 1995; Reynolds *et al.*, 1996; Farris *et al.*, 1999). La associates with ribonucleoproteins and Ro60 protein which may explain why autoantibodies to La appear with autoantibodies to Ro60 (see section 1.4.4). Additionally, La and Ro and found together in apoptotic blebs (Casciola-Rosen *et al.*, 1994a). Patients with anti - Ro and anti - La antibodies are more likely to have SS and cutaneous disease while patients with anti - Ro antibodies only are more likely to have renal and lung disease. Anti - La antibodies are IgG class switched and show somatic hypermutations suggesting that there is an underlying antigen driven T cell dependent process involved. This is further supported by the close association between the presence of anti - La antibodies and the possession of the HLA DR3 DQ2/6 haplotype (Bell and Maddison, 1980; Schur *et al.*, 1982; Reveille *et al.*, 1991). This data suggests that La has a role in the pathogenesis of SLE and SS. The hypothesis being examined in the present study is that La is a T cell autoantigen in SLE and SS.

T cell responses to recombinant La have been reported in healthy individuals and patients with SS (Halse *et al.*, 1996, Helsloot and Sturgess, 1997). However, there was no difference in the number of patients responding to the antigen compared with the healthy individuals. Since La specific T cells to whole antigen were not specifically identified in patients it is speculated in the present study that the La protein is modified prior to its presentation to T cells. The association between disease and the presence of serum anti - La and anti - Ro antibodies suggests that apoptosis plays a role in the processing of La protein to reveal neo - epitopes. To address this 132 overlapping peptides of 15 amino acids in length corresponding to the entire sequence of the La protein were synthesised.

Peptides of this nature should reveal most of the antigenic epitopes as well as any cryptic epitopes. Proliferative responses were examined to these synthetic peptides in the peripheral blood of healthy individuals and patients with SLE and / or SS in order to identify La specific T cells which are specific for disease.

6.1 Subjects

Five groups of subjects were identified for this study depending on the haplotype of the individual and clinical diagnosis (Table 6.1). Twenty patients with SLE and / or SS were selected. Fourteen patients had the HLA DR3 DQ2 haplotype. Eleven of the 14 showed positive serum anti - La antibody titres as determined by an anti - La antibody specific ELISA. The remaining 6 patients had the HLA DR1 or HLA DR4 haplotype and were negative for HLA DR3 and HLA DQ2 or 6 haplotypes. These patients did not have serum anti - La antibodies.

Twenty three healthy individuals were recruited from the laboratory or the West Midlands Blood Transfusion Service. Eight had the HLA DR3 DQ2 haplotype, seven had the HLA DR1 haplotype and were negative for the HLA DR3 and HLA DQ2 or 6 haplotype, and eight were HLA DR3 negative but HLA DQ2 or HLA DQ6 positive. All of the healthy individuals had a negative anti - La antibody titre (Table 6.1).

6.2 Proliferative response to standard antigens

Proliferative responses to PHA, tetanus toxoid, PPD and rabies protein were determined in all of the subjects using a 7 day proliferation assay (see section 3.3.9). Proliferative responses for each individual are given in Figure 6.1 and Appendix A. A proliferative response was considered positive if the stimulation index was greater than, or equal to, 3. All of the 23 healthy individuals showed a proliferative response to PHA, tetanus toxoid and PPD, except for GG who did not respond to tetanus toxoid. Eight healthy individuals responded to rabies protein. The response to medium ranged between 155 CPM and 930

Table 6.1

	D.O.B	Diagnosis	HLA DR	HLA DQ	anti-La titre
DR3 healthy individuals					
PB*	27.6.69		0301/4	0201/2	1.3
JF*	4.2.67		0301/4	0201/2	1.3
PK	21.6.49		1501, 0304	0601/2, 0201/2	1.5
JM*	10.7.65		0301/4, 1301/16	0201/2, 0603	1.5
DP*	30.6.64		0301/4, 1301/16	0201/2, 0603/14	1.3
PS			0301/4	0201/2	1.5
HU	17.4.47		0301/4, 04	0201/2, 0302/7/8	1.6
BW	18.2.48		0301, 1401/7	0201/2, 0301/4	1.5
DR3 patients					
PBR		SS	0301, 0801-11	0201/2, 0401/2	> 100
GC	2.6.38	SLE 1986	1501, 0301	0201/2, 0601/2	1.9
HCO	28.6.73	SLE 1989	0101, 0301	0201/2, 0501-4	3.9
JC	24.4.45	SLE 1987	0301, 0701	0201/2	19.8
BF	27.6.44	SLE 1988	0301, 1301	0201/2, 0603	1.2
HJ	18.12.73	SLE 1993	1501, 0301	0201/2, 0602	2.3
HK		SS 1997	0301, 08	0201/2, 0301/4	> 100
AL	22.12.37	SLE 1968	*0301	0201/2	16.6
BM		SS	1501/6, 0301/4	0201/2, 0602/10	> 100
JMA	12.3.73	SLE/SS 1989	0801-11, 0301	0201/2, 0401/2	24.9
APO	26.6.44	SLE 1983	0301, 1302	0201/2, 0604-9	7.4
DP	1.8.49	SLE 1978	11, 0301	0201/2, 0301/4	54.5
JP	22.8.47	SLE 1980	*0301	0201/2, 0603	10.5
DW	4.12.65	SLE/SS 1992	1302, 0301	0201/2, 0604-9	0.7
DR1 healthy individuals					
GBA	3.2.56		0101/5, 1101	0501-4, 0301	1.3
BC	2.6.65		0101/2/4	0501-4	0.3
DC*	11.2.65		1, 14	*05	0.4
GG	18.7.62		0101/5, 1101	0501-4, 0301	0.3
DJ	3.3.67		0101/5	0501-4	0.3
JK	8.7.53		0101/5, 08	0401/2, 0501-4	nd
CR	24.4.71		*0103	*05	0.3

Table continued overleaf

Table 6.1 continued

	D.O.B	Diagnosis	HLA DR	HLA DQ	anti-La titre
DR1/4 patients					
KA	21.6.76	SLE/APS 1995	0101/2/4, 0401-22	05, 0301/4	0.3
PBA	6.8.46	SLE 1963	0401, 0404	*0302	0.4
KD	21.5.64	SLE 1981	0101, 0401-22	0501-4, 0301/4	0.4
SH	6.9.46	SLE 1989	0401/4	0301/4, 0302	0.4
LJ	19.2.64	SLE 1981	0101/2/4, 0401	05, 0302	0.3
CM	1.7.47	SLE 1984	0401, 0408	0301/4, 0302	0.4
DQ2/6 healthy individuals					
GB	20.7.44		0101/5, 1302	0501-4, 0604-9	nd
CC*	2.6.72		16, 0701/3	0201/2, 05	0.4
MD	25.10.71		15, 0701	02, 0602	0.3
AG	21.10.51		1501, 0401-22	0602, 0301/4	0.3
SH	17.12.68		1302, 1201-3	0604-9, 0301/4	nd
CL	3.4.56		0101/5, 1302	*0604	0.3
ML	26.10.40		0102, 0701	0201/2, 0501-4	0.3
PW*	15.1.61		11, 1302	0604, 0301	0.3

Table 6.1

Subjects (* denotes male) enrolled into the study with the date of birth (D.O.B.), clinical diagnosis, HLA DR and HLA DQ haplotype and serum anti - La antibody titre for each individual.

Five groups were established:

- healthy individuals with HLA DR3 DQ2 haplotype
- SLE and / or SS patients with HLA DR3 DQ2 haplotype
- healthy individuals with HLA DR1 haplotype
- SLEand / or SS patients with HLA DR1 or 4 haplotype
- healthy individuals with HLA DQ2 or DQ6 but not HLA DR3

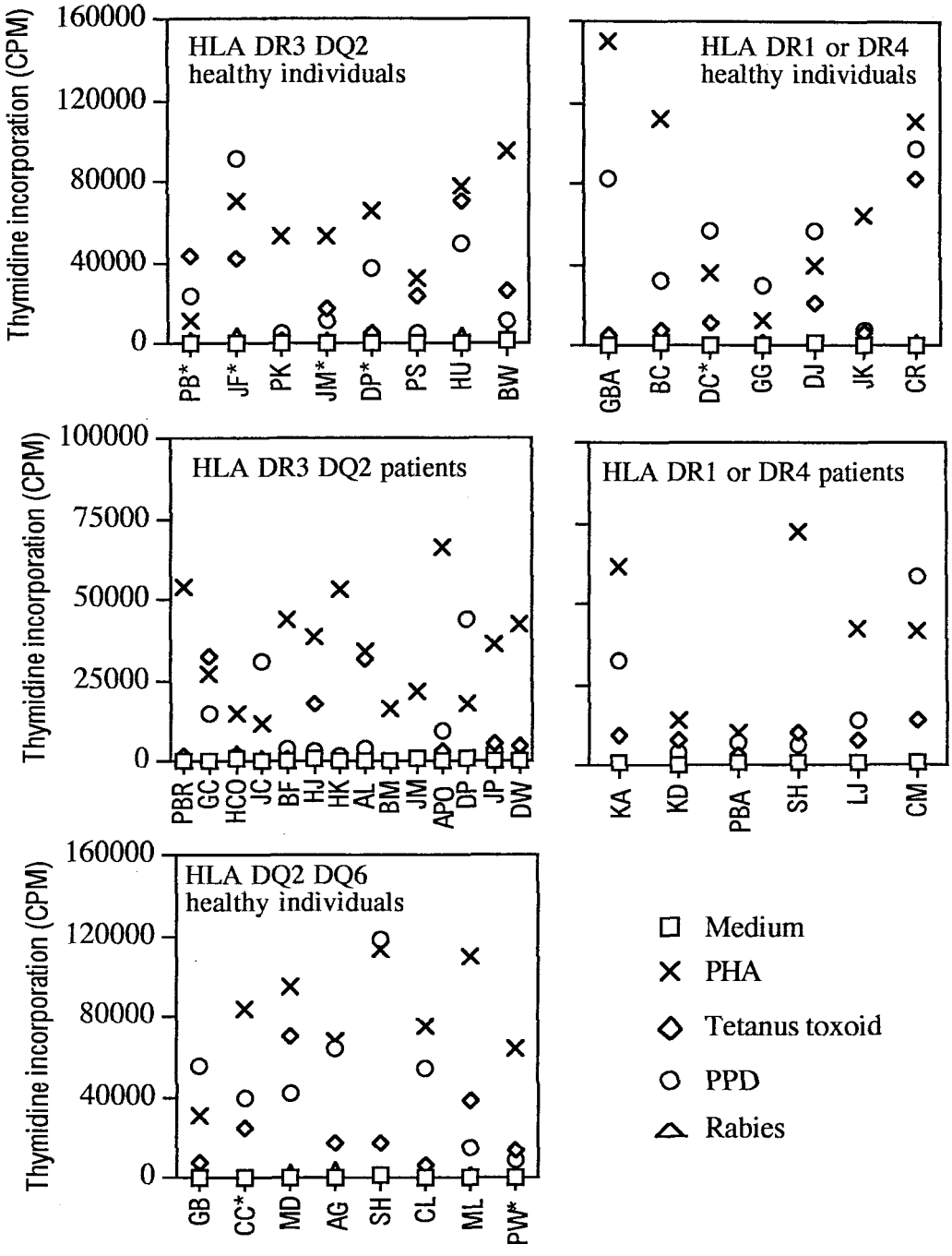


Figure 6.1

Proliferative responses (tritiated thymidine incorporation) in healthy individuals and patients with the HLA DR3 DQ2 haplotype, HLA DR1 or DR4 haplotype or HLA DQ2 or DQ6 haplotype.Triplicate cultures of $2x10^5$ cells were incubated for 7 days with either tetanus toxoid, purified protein derivative (PPD), rabies protein or PHA (see section 3.3.9). The results are expressed as mean CPM of the triplicates.

CPM. All of the 20 patients showed a proliferative response to PHA. Twelve patients responded to both tetanus toxoid and PPD, a total of five responded to either tetanus toxoid alone or PPD alone and three did not respond to either tetanus toxoid or PPD (HCO, BM and JMA). Five of the 20 patients showed a proliferative response to rabies protein (BF, HJ, AL APO and KD). The response to medium ranged between 163 CPM and 986 CPM.

6.3 Proliferative response to La peptide pools

The proliferative response to 15mer peptides covering the entire length of the La protein was examined using a standard 7 day proliferative assay (see section 3.3.9). Owing to the large number of peptides to be examined (132 in total) and the limited number of PBMC's obtained from patients an initial screening method was developed to identify stimulatory peptides. The La peptides were divided into 14 pools with the start of each peptide in a pool shifted along six amino acids from the previous (see Appendix B). Pool 1 (amino acid range 4 - 72) and pool 8 (amino acid range 1 - 69) covered the same sequence of the La protein except for a shift of 3 amino acids. Likewise, pools 2 and 9, 3 and 10, 4 and 11 etc covered the same sequence but with a shift of three amino acids. Each peptide in the pool was used at a final concentration of 10μg/ml. In addition, two control peptides were examined. A tetanus toxoid peptide spanning amino acids 830 - 844 was used, as it binds most HLA DR molecules (Reece *et al.*, 1994), and a Hsp65 (*Mycobacterium leprae*) peptide spanning the first 15 amino acids was used as it specifically binds HLA DR3 molecules (Gaston *et al.*, 1990). Between 5 and 14 replicate wells were set up in culture for each of the peptide pools to ensure a significant number of peptide specific T cells were present. The number of replicate wells established varied between subjects and was dependent on the number of PBMC's obtained. Anti - CD28 monoclonal antibody was added to the culture unless otherwise stated. Results were examined as the mean CPM value of the replicate wells or as the percentage of wells giving a positive response. Individual CPM values are given in Appendix C.

6.3.1 HLA DR3 DQ2 Subjects

Proliferative responses to 14 La peptide pools were examined in 8 healthy individuals and 14 patients with the HLA DR3 DQ2 haplotype. Anti - La antibodies were absent in the serum of healthy individuals, but present in 11 of the 14 patients although low in 3 of the positive patients (Table 6.1). Initial data analysis was performed using the mean CPM values for each of the peptide pools. Mean proliferative responses equal to or greater than a stimulation index of three were considered as positive (SI ≥ 3). Figure 6.2 shows a representative proliferative response in one healthy individual (JM) and one patient (BF) to the peptide pools. Individual JM responded to pools 1, 5 and 8. Patient BF showed a proliferative response to peptide pools 1, 3 and 8. Table 6.2 summarises the mean proliferative responses to the peptide pools in the eight healthy individuals and 14 patients studied. Only peptide pools stimulating a response with a SI ≥ 3 are listed. Individual CPM values are given in Appendix C. Each subject showed a unique pattern of proliferation in response to the peptide pools. However, peptide pools 1, 3, 5, 8 and 10 were identified as pools which predominantly stimulated a proliferative response in the subjects (Figure 6.3). There was no association between the proliferative response to the peptide pools and the presence of serum anti - La antibodies or the ability to respond to standard antigens.

The magnitude of the proliferative responses to the peptide pools was similar in healthy individuals and patients. Responses to pool 1 were found in healthy individuals PB and JM (SI = 3.3 and 3.1, respectively) and patients JP, HCO, DP, BF and GC (SI = 4.0, 4.4, 3.5, 9.6 and 4.5, respectively). Responses to pool 3 were found in patients APO, HJ, HK, BM, DW and BF (SI = 3.1, 3.5, 3.1, 3.5, 3.3 and 9.2 respectively). Responses to pool 5 were found in healthy individuals PK, BW and JM (SI = 4.6, 10.7 and 3.8, respectively) and patients AL and DW (SI = 3.5 and 3.6 respectively). Responses to pool 8 were found in healthy individuals PS, BW and JM (SI = 4.9, 10.7 and 5.7, respectively) and patients APO, HJ, DP, AL, BF, HK, JC and PBR (SI = 5.0, 4.9, 4.2, 3.5, 4.2, 5.0, 3.3 and 3.0, respectively). Responses to pool 10 were found in healthy individual PK (SI = 3.4) and patients HJ and GC (SI = 8.2 and 3.7, respectively).

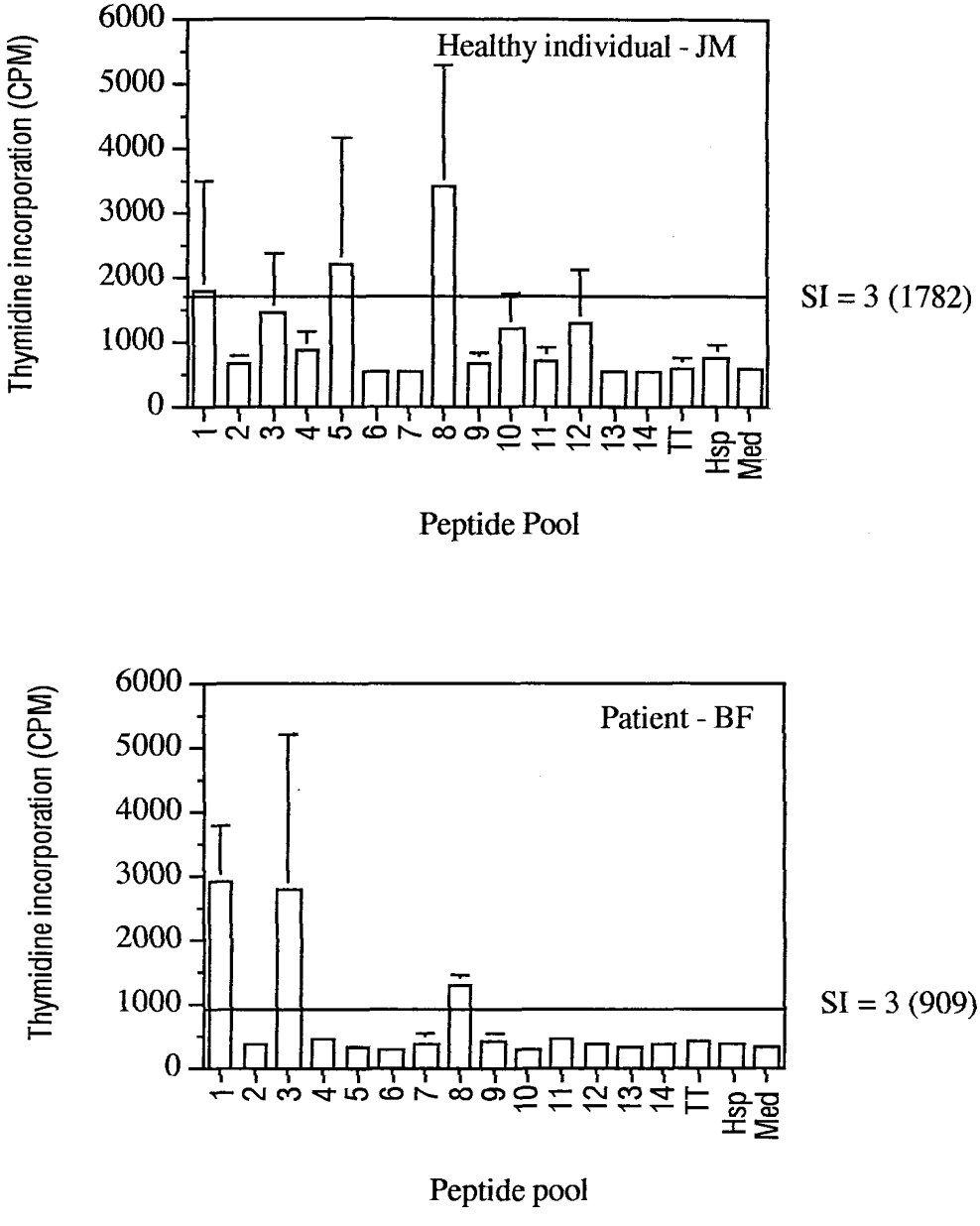


Figure 6.2

Representative proliferative responses (tritiated thymidine incorporation) obtained from one HLA DR3 DQ2 healthy individual and one HLA DR3 DQ2 patient with SLE. $2x10^5$ fresh PBMC's were incubated for 7 days with either the La peptide pools (1 - 14), control peptides of tetanus toxoid 830 - 844 (TT) or heat shock protein 65 (Hsp) or medium (med). The results are expressed as mean CPM of the replicate wells ± standard deviation. The horizontal line indicates a positive response when the stimulation index equals 3.

Table 6.2

	Mean positive response	At least 20% wells positive	At least 50% wells positive	At least 80% wells positive	No. of wells
DR3 healthy individuals					
PB*	1, hsp	hsp	hsp		6
JF*	hsp	10, hsp	hsp		14
PK	5, 10	5, 10	5		10
JM*	1, 5, 8	1, 3, 5, 8, 12	8	8	10
DP*					14
PS	8	5, 8, hsp	8		5
HU	hsp	1, 3, 8, hsp	hsp	hsp	9
BW	5, 8, hsp	1, 5, 8, 10, hsp	5, 8, hsp	5, hsp	10
DR3 patients					
PBR	8	8			6
GC	1, 10, hsp	1, 10, hsp	1, 10, hsp	1, hsp	6
HCO	1	1, 3	1		6
JC	8	8, 10	8, 10	8	6
BF	1, 3, 8	1, 3, 8	1, 3, 8	1, 3, 8	6
HJ	3, 8, 10	1, 3, 8, 10, hsp	1, 3, 8, 10	8, 10	11
HK	3, 8	1, 3, 8	3, 8	8	6
AL	3, 5, 8	3, 5, 8	8	8	6
BM	3	3	3	3	10
JM	4	4, hsp	4, hsp		5
APO	3, 8	3, 8, 10	3, 8	8	6
DP	1, 5, 8, hsp	1, 5, 6, 8, 12, hsp	5, 8, hsp		14
JP	1, 5	1			9
DW	3, 5, 8, 12	1, 3, 5, 6, 8, 12	3, 5, 8, 12	3, 5, 8, 12	5
DR1 healthy individuals					
GBA					5
BC		10			6
DC*					8
GG	1, 5, 12	1, 5, 12			10
DJ	1	1	1		8
JK	1, 5	1, 5	1		6
CR					5

Table continued overleaf

Table 6.2 continued

	Mean positive response	At least 20% wells positive	At least 50% wells positive	At least 80% wells positive	No. of wells
DR1/4 patients					
KA					5
PBA					5
KD					5
SH					5
LJ	3	3, 4, 12			6
CM					5
DQ2/6 healthy individuals					
GB	5, 12	5, 12	5, 12	5	6
CC*					8
MD					14
AG	1, 5, 8, 10	1, 5, 8, 10	1, 5, 8	1, 5, 8	6
SH					5
CL	1, 5, 8, 12	1, 4, 5, 8, 10, 12	1, 5, 8, 12	1, 5, 12	8
ML	1, 8, TT	1, 3, 8, 10, TT	1, 8, TT		14
PW*	1, 8, 10	1, 3, 8, 10	1, 8, 10	10	10

Table 6.2

Summary of proliferative responses in patients and healthy individuals to the 14 La peptide pools. The second column 'mean positive response' indicates a mean proliferative response in the replicate wells. The following three columns indicate whether a response was found in at least 20%, 50% or 80% of the wells cultured. The last column, number of wells, indicates the number of replicate wells established for each subject to each of the peptide pools. A positive response was considered as any well with a stimulation index of three or more.

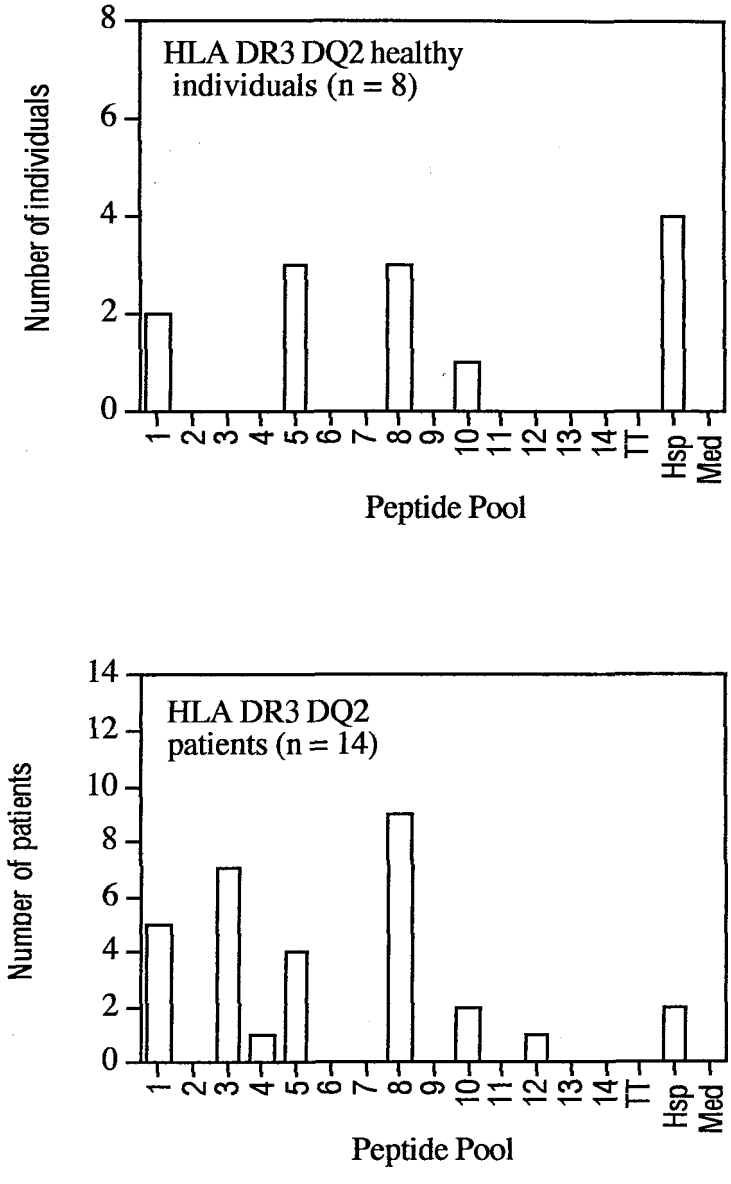
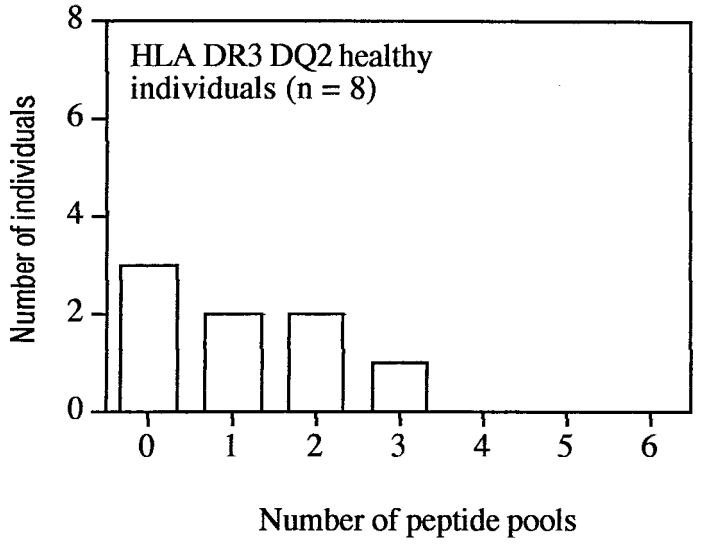


Figure 6.3

Number of healthy individuals and patients with SLE, both with the HLA DR3 DQ2 haplotype, showing a positive response (stimulation index ≥ 3) to the La peptide pools (1 - 14) and control peptides tetanus toxoid 830 - 844 (TT) and heat shock protein 65 (Hsp).

Figure 6.4 shows the number of healthy individuals and patients and the number of peptide pools they responded to. Figure 6.4 also shows that two healthy individuals responded to only one pool (PS and PB), two individuals responded to two pools (BW and PK) and one individual responded to three pools (JM). Three healthy individuals failed to show a proliferative response to any of the peptide pools although they proliferated in response to PHA and PPD stimulation. Five patients respond to only one pool, four patients responded to two pools or three pools and one patient responded to four pools. Four healthy individuals (BW, HU, JF and PB) and 2 patients (GC and DP) responded to the control Hsp65 peptide and one patient (HJ) responded to the tetanus toxoid control peptide.

Analysing the proliferation responses to the La peptide pools in terms of the mean CPM value allows regions of the La protein stimulating a proliferative response to be identified. However, the standard deviations were large indicating that some of the replicate wells failed to respond to the peptide pool. Using PBMC's from responding individuals, tetanus toxoid stimulates a positive proliferative response (SI $\geq$ 3) in 100% of the wells cultured with antigen with a standard deviation of approximately 10% of the mean CPM value. However, in response to rabies protein only 30 - 50% of the wells cultured with the antigen are positive (SI $\geq$ 3) although the overall mean CPM has a SI $\geq$ 3. The standard deviations are up to 80% of the mean CPM value (see Appendix C). A low percentage of positive responding wells therefore suggests a naive T cell response and a high percentage of responding wells suggests a primed T cell response. Figure 6.5 shows the percentage of positive wells in one healthy individual (JM) and one patient (BF). Healthy individual JM responded to peptide pools 1, 5, 8 and 12 in which at least 20% were positive and to peptide pool 8 in which at least 80% of the wells were positive. Patient BF responded to peptide pools 1, 3 and 8 in which at least 50% of the wells were positive and to peptide pools 1 and 8 in which at least 80% of the wells are positive. Table 6.2 and Figure 6.6 summaries for the 8 healthy individuals and 14 patients the peptide pool responses in which at least 20%, 50% and 80% of the replicate wells were positive. Proliferative responses occur to the same pools in both healthy individuals and patients (pools 1, 3, 5, 8, 10 and 12). However, the patients responded to the peptide pools in a greater percentage of



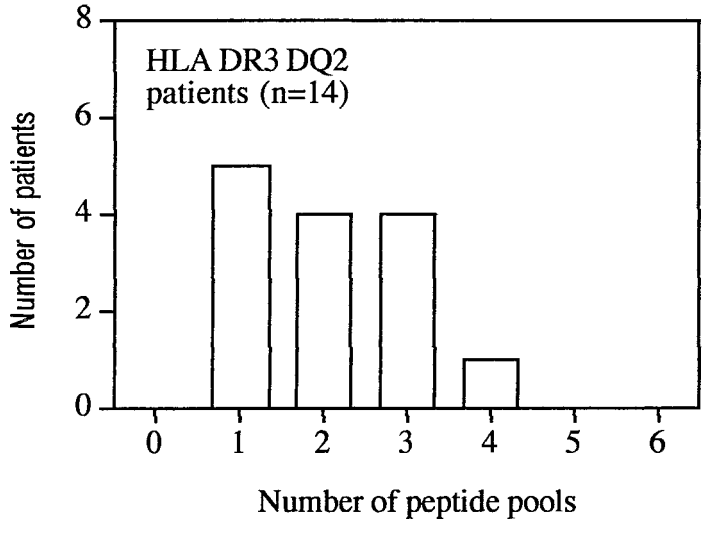


Figure 6.4

The number of healthy individuals and patients with SLE, both with the HLA DR3 DQ2 haplotype showing how many different peptide pools they respond to with a SI ≥ 3.

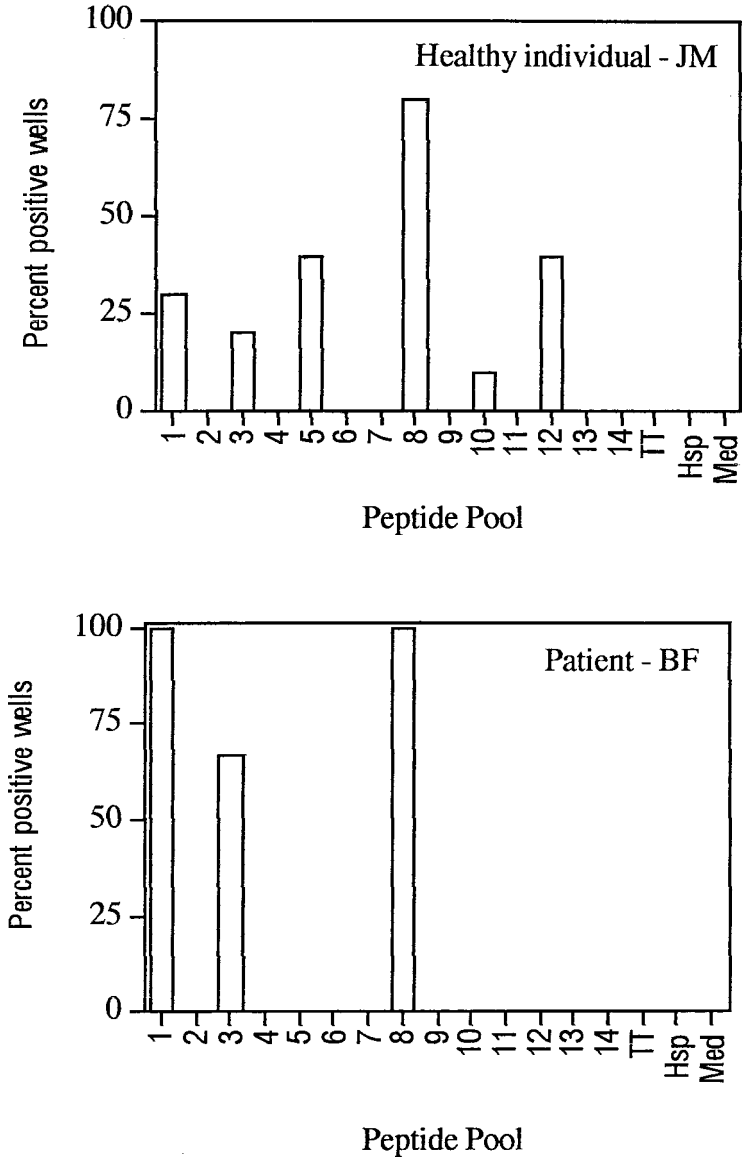


Figure 6.5

Percent positive wells (stimulation index ≥ 3) obtained from a HLA DR3 DQ2 healthy individual (JM) and patient with SLE (BF) in response to the La peptide pools (1 - 14), control peptides tetanus toxoid 830 - 844 (TT) and heat shock protein 65 (Hsp) or medium (med). For JM ten replicate wells were established for each peptide pool. For BF six replicate wells were established for each peptide pool.

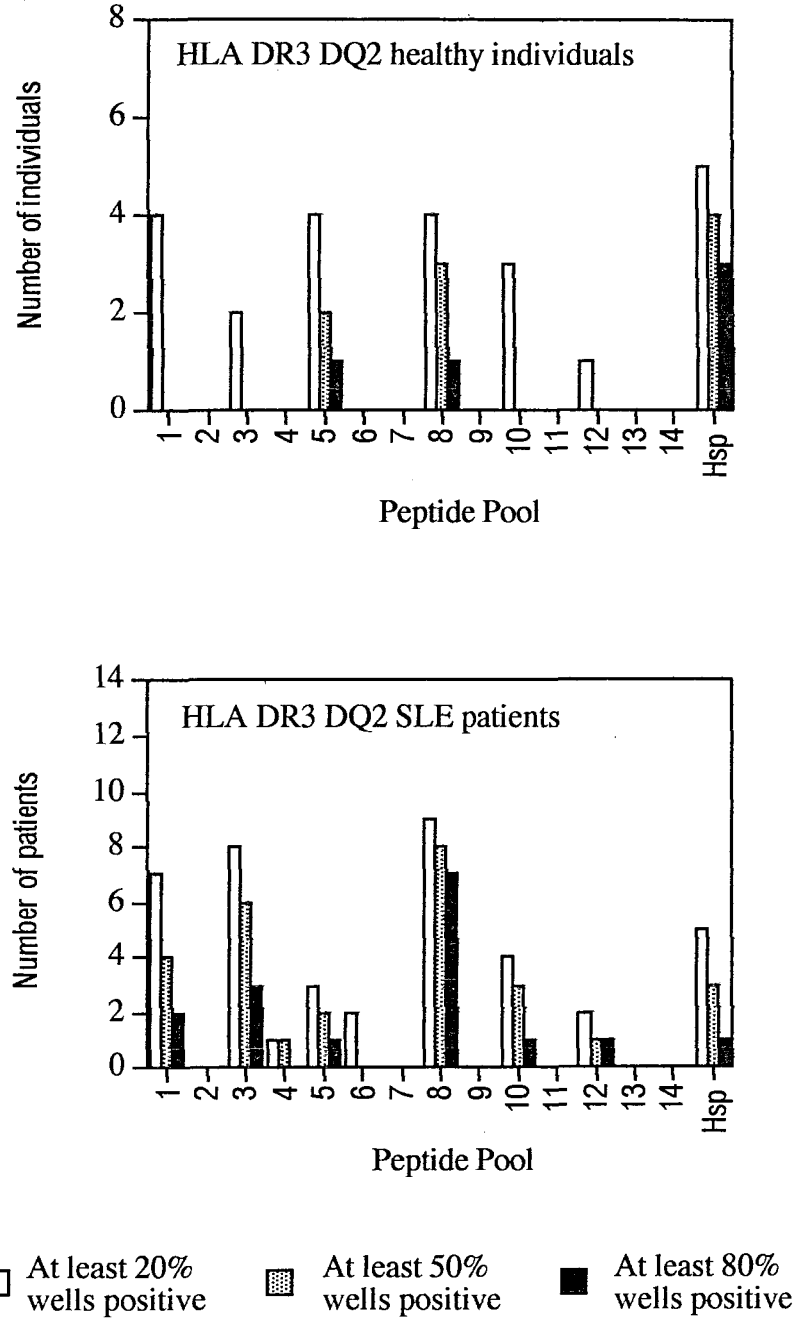


Figure 6.6

The number of healthy individuals and patients with SLE, both with the HLA DR3 DQ2 haplotype showing a positive response (SI ≥ 3) to the La peptide pools (1 - 14) and control peptide heat shock protein 65 (Hsp). See Table 6.2 for the number of replicate wells established for each subject to each of the peptide pools.

the replicate wells (Figures 6.6 and 6.7).

6.3.2 HLA DR1 or DR4 Subjects

As summarised in Table 6.2, four of the seven healthy individuals with the HLA DR1 haplotype studied failed to show a proliferative response to any of the peptide pools. Responses were found to peptide pools 1, 5 and 12 when analysing the data as the mean CPM value of the replicate wells. Stimulation index values ranged between 3.1 and 4.7. However, responses were observed in 50% or fewer of the replicate wells cultured. Of the six patients studied only one patient (LJ) showed a proliferative response to the peptide pools. A response was seen to pool 3 using the mean CPM value (SI = 3.5) but 20% of the wells cultured with pools 3, 4 and 12 also showed a positive proliferative response. None of the subjects responded to the tetanus toxoid or the Hsp65 control peptides. A control peptide binding only HLA DR1 was unavailable during the course of the study to test the function of HLA DR1 molecules in the subjects.

6.3.3 HLA DQ2 or DQ6 healthy individuals

Three of the 8 healthy individuals failed to respond to any of the peptide pools (Table 6.2). Proliferative responses were found to peptide pools 1, 5, 8, 10 and 12. Stimulation index values ranged between 3.4 and 8.2 and responses were found in at least 80% of the replicate wells. One healthy individual (ML) responded to the control tetanus toxoid peptide but none responded to the Hsp65 peptide.

6.3.4 Reproducibility of peptide responses

As the proliferative responses to the peptide pools are small the reproducibility of the responses was examined by repeating the experiment three months after the initial experiment. Two HLA DR3 DQ2 healthy individuals (JF and PB) and two patients (GC and HJ) were studied. Figure 6.8 shows the proliferative responses in one healthy

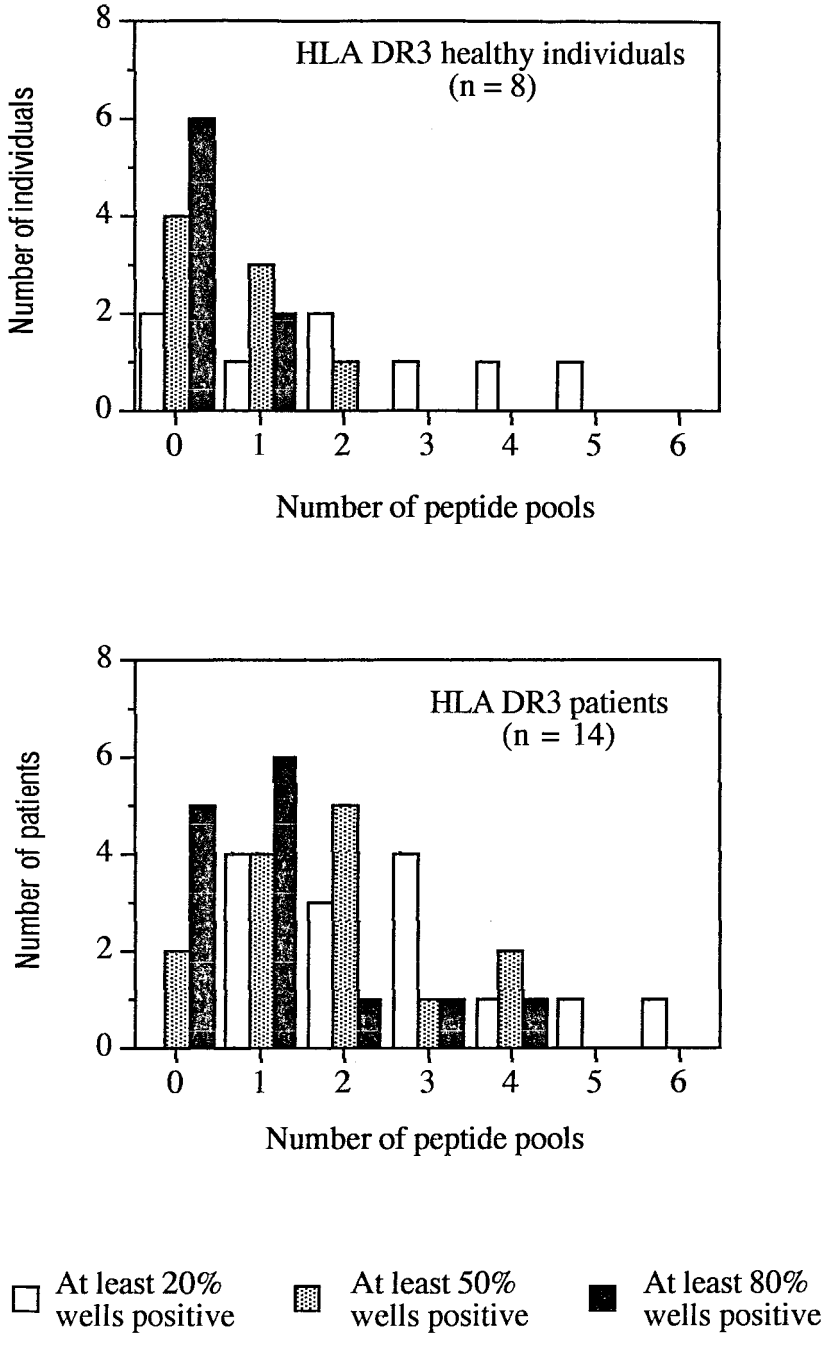


Figure 6.7

The number of healthy individuals and patients with SLE, both with the HLA DR3 DQ2 haplotype, showing a positive response (SI ≥ 3) in at least 20%, 50% or 80% of the wells cultured with each of the La peptide pools (1 - 14). See Table 6.2 for the number of replicate wells established for each subject to each of the peptide pools.

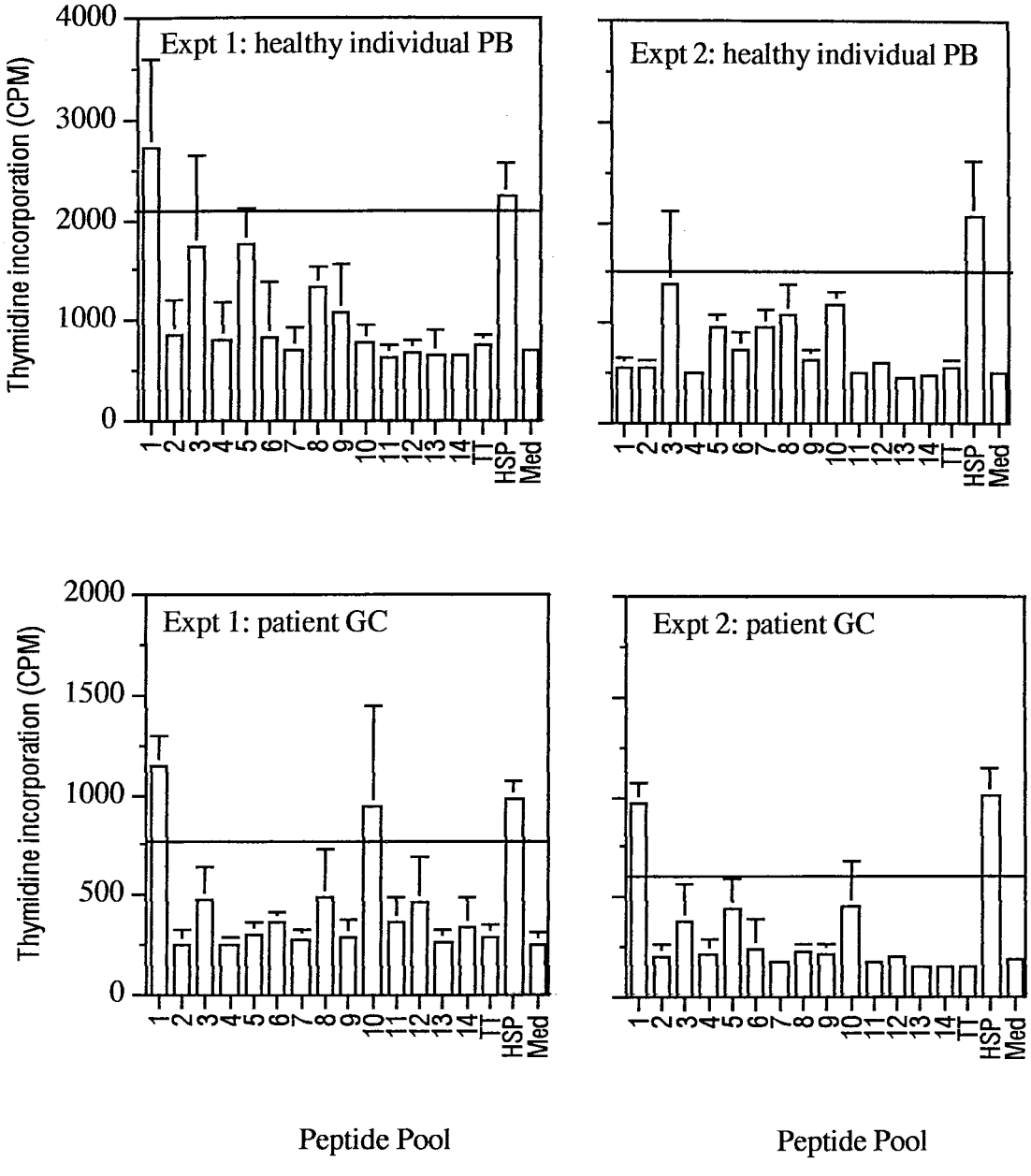


Figure 6.8

Proliferative response (thymidine incorporation) in a healthy individual and a patient to the La peptide pools in two separate experiments. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation. The horizontal line indicates a SI = 3.

individual and one patient but are representative of all the subjects tested. Healthy individual PB responded to peptide pool 1 and the Hsp65 control peptide but only to the Hsp65 control peptide in the repeat experiment. Patient GC responded to peptide pools 1, 10 and the Hsp65 control peptide but failed to respond to peptide pool 10 in the repeat experiment. However, even if a proliferative response did not reach a stimulation index of 3 the same pattern of responses was observed in the repeat experiments.

6.3.5 Peptide toxicity

To confirm that the inability of the non - stimulatory peptide pools to elicit a response was not due to a toxic effect each pool was tested in combination with tetanus toxoid (Figure 6.9). One healthy individual and one patient with the HLA DR3 DQ2 haplotype was studied. There was a small reduction in the proliferative response to tetanus toxoid when some of the peptide pools were simultaneously cultured with the antigen but the reductions were not significant.

6.3.6 Effect of anti - CD28 antibody on the proliferative response to the peptide pools

The effect of anti - CD28 monoclonal antibody on the proliferative responses to the peptide pools was examined since its addition may bias the proliferative response (see section 4.2). One healthy individual and one patient, both with the HLA DR3 DQ2 haplotype, were tested. The same pattern of response was seen with or without anti - CD28 monoclonal antibody (Figure 6.10). Anti - CD28 antibody had little effect on the magnitude of the proliferative response to the peptide pools in the healthy individual. In the patient the proliferative response to the pools was improved with anti - CD28 antibody although the same pools were stimulatory.

6.3.7 Effect of peptide concentration

To confirm that the peptide concentration used *in vitro* did not effect which peptide pools

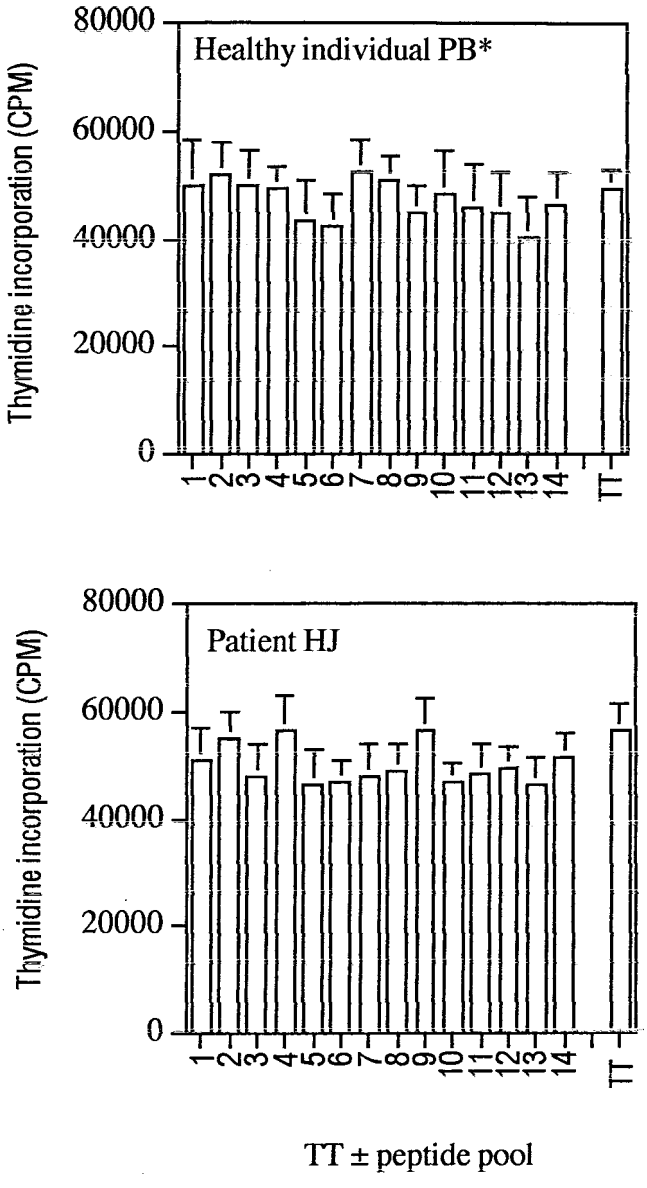


Figure 6.9

Proliferation responses (tritiated thymidine incorporation) in a healthy individual and a patient to tetanus toxoid (TT) with or without the addition of the La peptide pools. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation.

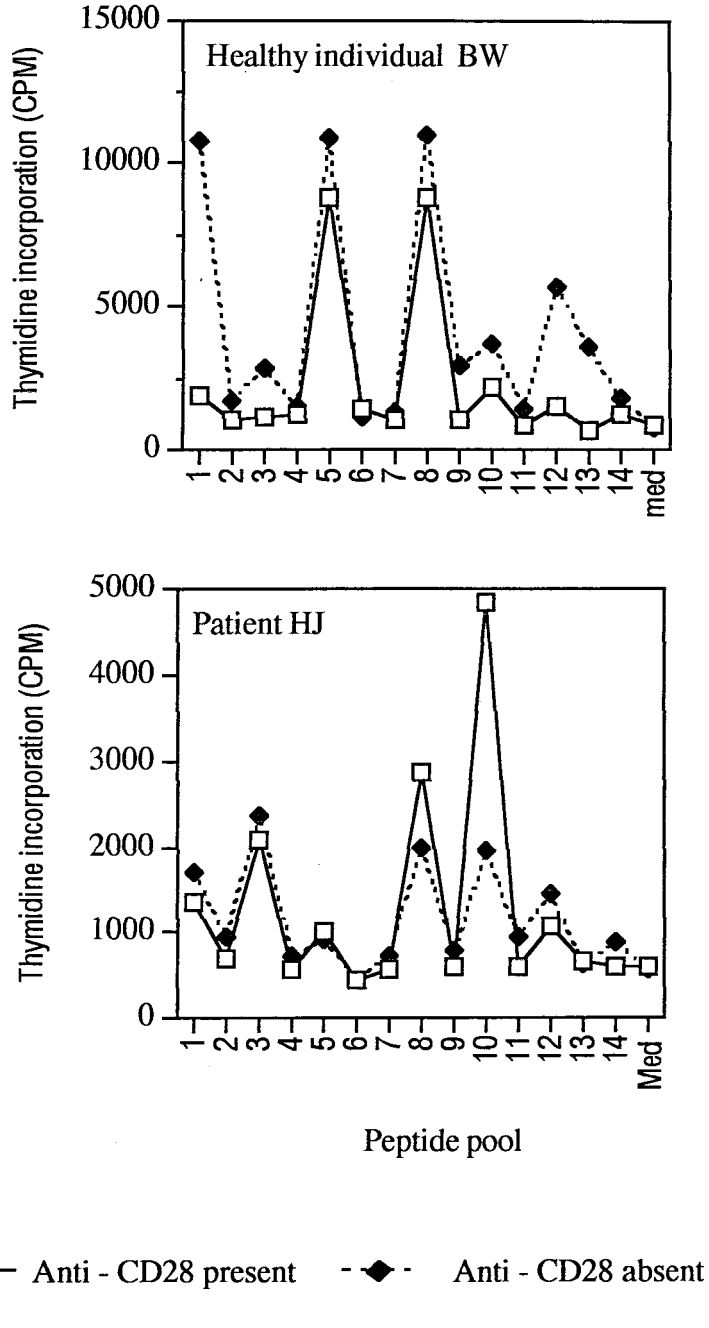


Figure 6.10

Proliferative responses (tritiated thymidine incorporation) obtained from one HLA DR3 DQ2 healthy individual and one HLA DR3 DQ2 patient. $2x10^5$ fresh PBMC's were incubated for 7 days with the La peptide pools in the presence or absence of anti - CD28 monoclonal antibody (see section 3.3.9). The results are expressed as mean CPM of the replicate wells.

were stimulatory, peptide pools were made containing either 1μg/ml or 10μg/ml of each peptide. The pools were tested in two healthy individuals with the HLA DR3 DQ2 haplotype (Figure 6.11). At 1μg/ml the proliferative response to the peptide pools was lost in both healthy individuals.

6.3.8 Responses to the La peptide pools are found in healthy individuals and patients

In summary, the proliferative response to the La peptide pools was examined in healthy individuals and patients with either the HLA DR3 DQ2, HLA DR1 or DR4, or HLA DQ2 or DQ6 haplotype. An initial screening method was devised in which the peptides were grouped into 14 pools (see Appendix B). Proliferative responses were predominant to 6 pools in subjects with the HLA DR3 DQ2 haplotype and in healthy individuals with the HLA DQ2 or DQ6 haplotype (Table 6.2). However, a unique pattern of response was seen for each subject. Responses occurred in more of the replicate wells in healthy individuals with the HLA DQ2 or DQ6 haplotype and patients with the HLA DR3 DQ2 haplotype compared to healthy individuals with the HLA DR3 DQ2 haplotype. Three of the 7 healthy individuals and one of the 6 patients with the HLA DR1 or DR4 haplotype responded to a peptide pool (pool 1, 5 or 12).

Proliferative responses to the peptide pools were not associated with serum anti - La antibody titres or the ability to proliferate in response to standard antigens (Tables 6.2 and 6.3 and Figure 6.1). The response was reproducible (Figure 6.8), lost when using 1μg/ml of each peptide (Figure 6.11) and, in patients, dependent on the presence of anti - CD28 monoclonal antibody (Figure 6.10).

6.4 Proliferative response to individual peptides

The aim of this section of work was to identify which of the individual peptides in the stimulatory pools, identified in section 6.3, were responsible for the proliferative responses. Since the proliferative responses to peptide pools 5 and 12 were weaker,

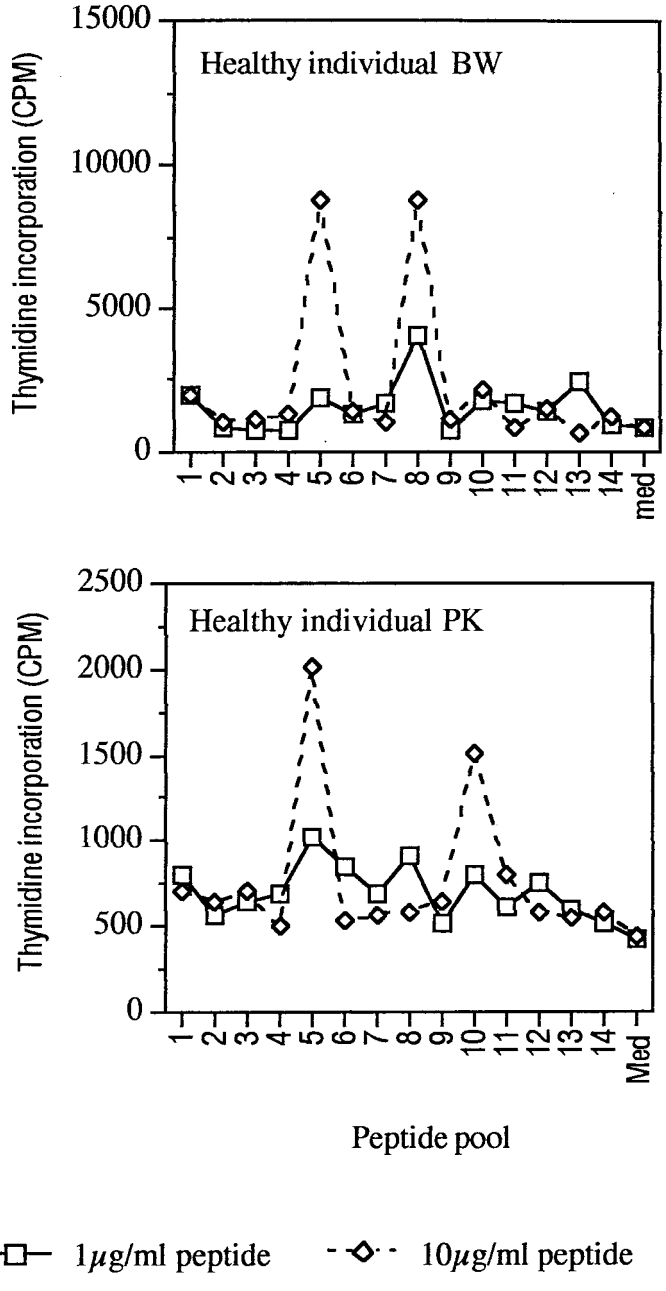


Figure 6.11

Proliferative responses (tritiated thymidine incorporation) obtained from two HLA DR3 DQ2 healthy individuals. $2x10^5$ fresh PBMC's were incubated for 7 days with the La peptide pools (see section 3.3.9). Each peptide in each pool was prepared at 1μg/ml or 10μg/ml. Anti - CD28 monoclonal antibody was present. The results are expressed as mean CPM of the replicate wells.

	Pool 1	Pool 8	Peptide 49 - 63	Human La	HLA DR	HLA DR	HLA DQ	HLA DQ	Anti-La Ab titre
DR3 healthy individuals									
PB	+	-	-	-	0301/4	0301/4	0201/2	0201/2	1.3
JF	-	-	+	+	0301/4	0301/4	0201/2	0201/2	1.3
JM	+	+	+	-	0301/4	1301/16	0201/2	*0603	1.5
DP	-	-	-	+	0301/4	1301/16	0201/2	*0603	1.3
PS	-	+	+	-	0301/4	0301/4	0201/2	0201/2	1.5
HU	-	-	-	-	0301/4	*04	0201/2	0302/7/8	1.6
DR3 patients									
GC	+	-	-	-	*0301	1501	0201/2	0601/2	1.9
HCO	+	-	+	+	*0301	*0101	0201/2	0501-4	3.9
JC	-	+	+	+	*0301	*0701	0201/2	0201/2	19.8
BF	+	+	+	+	*0301	1301	0201/2	*0603	1.2
HJ	-	+	+	+	*0301	1501	0201/2	*0602	2.3
AL	-	+	-	-	*0301	*0301	0201/2	0201/2	16.6
JMA	-	-	-	-	*0301	0801-11	0201/2	0401/2	24.9
APO	-	+	-	+	*0301	1302	0201/2	0604-9	7.4
JP	+	-	-	-	*0301	*0301	0201/2	*0603	10.5
DR1 healthy individuals									
GBA	-	-	-	-	1101	0101/5	0301-4	*0301	1.3
BC	-	-	-	-	0101/2/4	0101/2/4	0501-4	0501-4	0.3
GG	+	-	-	-	*1101	0101/5	0501-4	*0301	0.4
DJ	+	-	-	-	0101/5	0101/5	0501-4	0501-4	0.3
JK	+	-	-	-	*08	0101/5	0501-4	0401/2	nd
CR	-	-	-	-	*0103	*0103	*05	*05	0.3
DR1/4 patients									
PBA	-	-	-	-	*0401	*0404	*0302	*0302	0.4
SH	-	-	-	-	0401/4	0401/4	0301/4	*0302	0.4
LJ	-	-	-	-	*0401	*0101	*05	*0302	0.3
DQ2/6 healthy individuals									
GB	-	-	-	-	1302	0101/5	0501-4	0604-9	nd
MD	-	-	-	+	15	*0701	*02	*0602	0.3
AG	+	+	-	+	1501	0401-22	0301/4	*0602	0.3
CL	+	+	-	-	1302	0101/5	*0604	*0604	0.3
ML	+	+	-	+	*0701	*0102	0501-4	0201/2	0.3
PW	+	+	-	+	11	1302	*0604	*0301	0.3

Table 6.3

Summary of proliferative responses (SI ≥ 3) to peptide pools 1 and 8, peptide 49 - 63 and partially purified human La in healthy individuals and patients listing the haplotype and anti - La antibody titre for each individual.

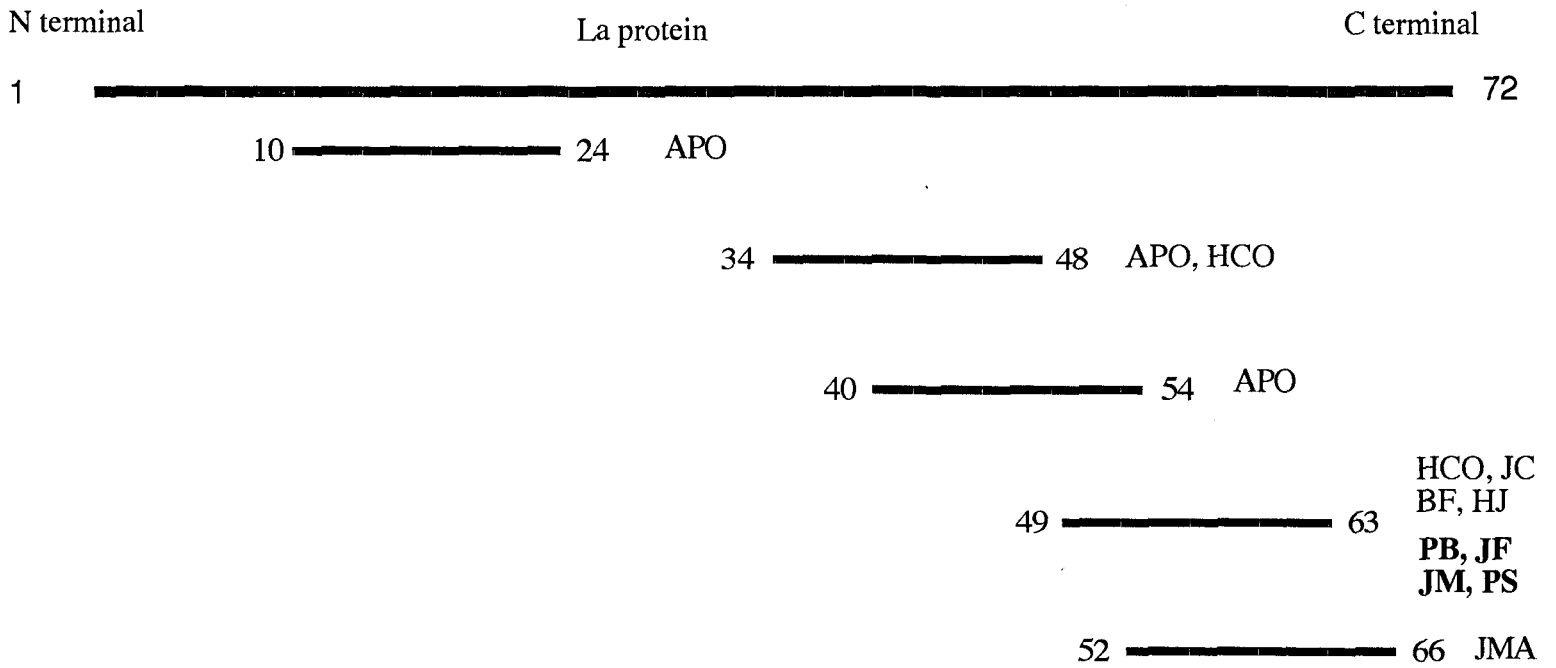


Figure 6.12

Summary of proliferative responses in healthy individuals (bold type) and patients (normal type) to 15mer peptides between amino acid 1 and 72 of the La protein. The peptides were all taken from peptide pools 1 and 8. The stimulatory peptides are marked showing which individual responded to the peptide with a mean CPM value having a SI ≥ 3.

compared with peptide pools 1, 3, 8 and 10, and occurred in subjects with the HLA DR1 or DR4 haplotype the attention was focussed on peptides comprising of pools 1, 3, 8 and 10. Forty 15mer peptides from pools 1 and 8 and 3 and 10 were therefore tested for their ability to stimulate a proliferative response in healthy individuals and patients using the 7 day assay. Unless otherwise stated anti - CD28 monoclonal antibody was added to all cultures and the peptides were used at 10μg/ml. A proliferative response was considered positive if the stimulation index (SI) was three times or more the response of medium alone. Individual CPM values are given in Appendix C.

6.4.1 Proliferative response to individual peptides taken from pools 1 and 8

Proliferative responses to 20 peptides covering amino acids 1 - 72 of the La protein were examined. The peptides were previously grouped into peptide pools 1 or 8. Table 6.3 and Figure 6.12 summaries the findings. Three of the six HLA DR3 DQ2 healthy individuals tested responded to peptide 49 - 63 (SI values ranged between 3.6 and 5.5 and the percentage of positive replicates ranged between 33% and 100%). JF did not respond to either of the peptide pools 1 or 8 which contained this 15mer but responded to the peptide 49 - 63. Responses were also found to peptides 4 - 18, 13 - 27, 16 - 30, 22 - 36, 37 - 51, 40 - 54, 52 - 66 but in fewer than 30% of replicate wells (see Appendix C for individual CPM values).

Of the 9 patients with the HLA DR3 DQ2 haplotype studied for responses to the 20 individual peptides one failed to respond to peptide pools 1 and 8 (JMA). However, this patient, JMA, responded to peptide 52 - 66. Proliferative responses (SI $\geq$ 3) are summarised in Table 6.3 and Figure 6.12. Stimulation index values ranged between 3.1 and 7.9. Four patients (HCO, JC, BF and HJ) responded to peptide 49 - 63 in which greater than 80% of the replicate wells were positive. Patients AL, JP and GC failed to show a proliferative response with a mean CPM value having a SI $\geq$ 3 to any of the 20 peptides. However, these patients showed a proliferative response to either peptide 7 - 21, 13 - 27, 22 - 36, 37 - 51 or 46 - 60 in less than 30% of the replicate wells.

As subjects with the HLA DR1 or DR4 haplotype did not respond to peptide pools 1 or 8 the proliferative response to the individual peptides was examined in only two subjects. One healthy individual (CR) and one patient (SH) failed to respond to any of the 20 individual peptides. However, the proliferative response to peptide 49 - 63 was examined in 12 of the 15 HLA DR1 or DR4 subjects since this peptide was stimulatory in subjects with the HLA DR3 DQ2 haplotype. All 12 subjects failed to respond to this peptide.

The proliferative response to the 20 individual peptides taken from peptide pools 1 and 8 were tested in three healthy individuals with the HLA DQ2 or DQ6 haplotype (AG, PW and ML). All three healthy individuals failed to show a response to any of the 20 peptides. The remaining five individuals were not tested for response to the 20 individual peptides either because they failed to respond to the peptide pools or were unavailable for further study. However, six of the 8 healthy individuals were tested for a proliferative response to peptide 49 - 63. All six individuals failed to respond to the peptide.

6.4.2 Proliferative response to individual peptides taken from pools 3 and 10

The proliferative response to 20 peptides covering amino acid 121 - 192 of the La protein were examined. The peptides were previously grouped into peptide pools 3 or 10. Four healthy individuals and 8 patients with the HLA DR3 DQ2 were tested. None of the subjects, except JC, responded to any of the 20 peptides in any of the replicate wells. Patient JC only responded to peptide 121 - 135 but in only 20% of the wells cultured (see Appendix C). The proliferative response to the individual peptides was also examined in patient SH (HLA DR4), healthy individuals CR (HLA DR1) and healthy individuals ML, AG and PW (HLA DQ2 or DQ6). None of these subjects, except AG, responded to any peptide in any of the replicate wells. Individual AG responded to peptide 151 - 165 in 50% of the replicate wells. Individual CPM values are given in Appendix C.

6.4.3 Peptide toxicity

To confirm that the lack of response to a peptide was not due to a toxic effect of the peptide the 40 peptides which comprised of peptide pools 1, 3, 8 and 10 were cultured with tetanus toxoid. The peptides had no significant effect on the response to tetanus toxoid (Figure 6.13).

6.4.4 The La peptide 49 - 63 is stimulatory in HLA DR3 DQ2 subjects

In summary, proliferative responses were examined to 40 amino acids corresponding to peptide pools 1 and 8 (amino acid range 1 - 72) and peptide pools 3 and 10 (amino acid range 121 - 192). Healthy individuals and patients with the HLA DR3 DQ2 haplotype responded predominantly to peptide 49 - 63 (Table 6.3 and Figure 6.12). The stimulation index and percentage of positive replicate wells were similar in both sets of subjects in response to peptide 49 - 63 (see Appendix C for individual CPM values). Subjects with the HLA DR1 or DR4, or HLA DQ2 or DQ6, haplotype failed to respond to this peptide although some of the subjects responded to peptide pool 1 (Table 6.3). The lack of response was not due to toxic effects of the peptides (Figure 6.13). Individual 15 mer peptides covering amino acid 121 - 192 failed to stimulate a mean proliferative response in any of the individuals tested (SI $\geq$ 3).

6.5 Proliferative response to peptide 49 - 63

Initial scanning of the entire La sequence revealed two peptide pools (amino acid range 1 - 72 and 121 - 192) which stimulated a strong proliferative response in healthy individuals and patients with the HLA DR3 DQ2 haplotype. Although the stimulation index was similar in the peptide pools between healthy individuals and patients responses were found in fewer replicate wells in healthy individuals compared to patients. Subsequent analysis of the individual peptides taken from the stimulatory pools showed that peptide 49 - 63 elicited a strong proliferative response in healthy individuals and patients with the HLA

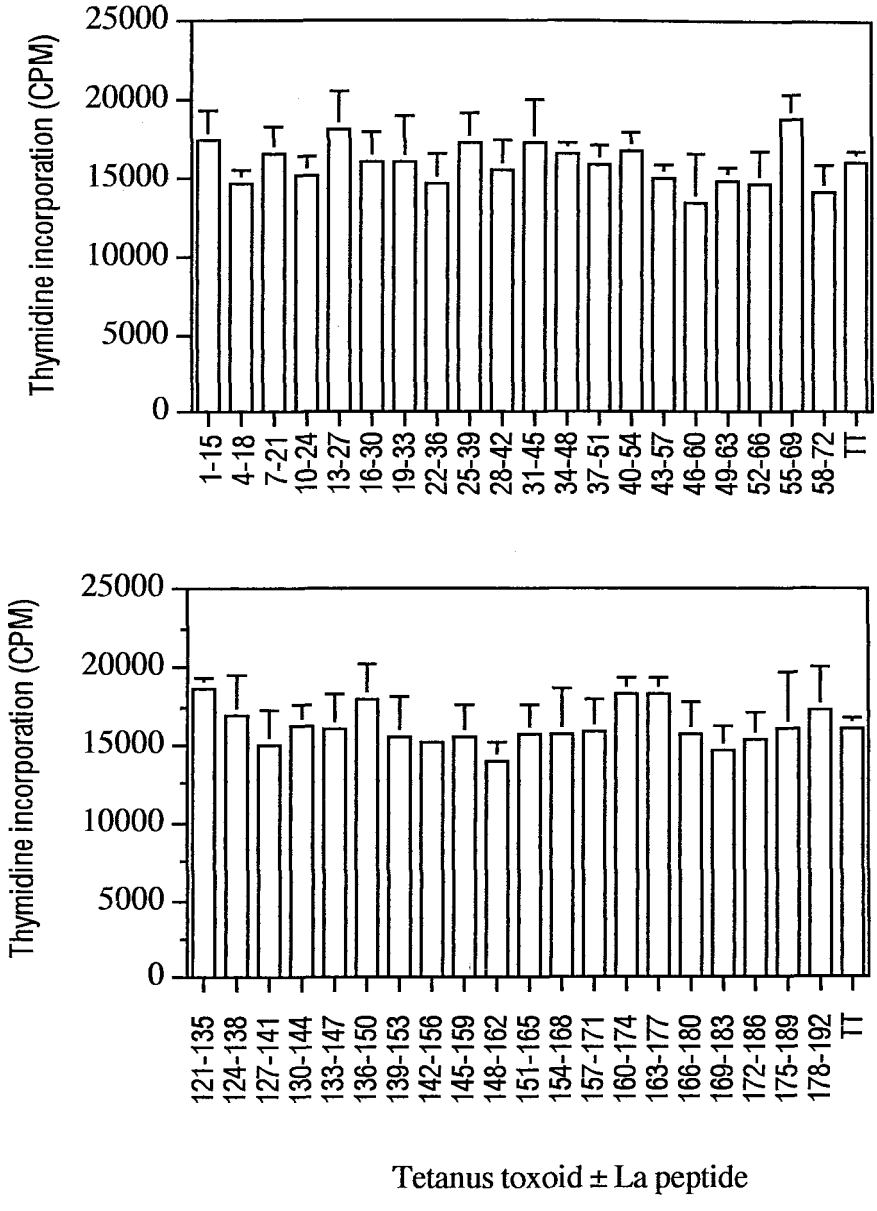


Figure 6.13

Proliferation responses (tritiated thymidine incorporation) in a healthy individual to tetanus toxoid (TT) and tetanus toxoid plus the La peptides. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation.

DR3 DQ2 haplotype. The stimulation index values were similar in healthy individuals and patients. However, comparing this response to tetanus toxoid and rabies protein suggested that the response to the peptide pools was mediated by naive T cells in healthy individuals and by primed T cells in patients. Additionally, the response to peptide 49 - 63 appeared to be mediated by the same T cell population in healthy individuals and patients. This suggestive data lead to analysing the response in CD45RA (naive) and CD45RO (primed) T cells and to examine if the response was restricted by the MHC.

6.5.1 Proliferative response to an alternative source of peptide 49 - 63

To confirm that the proliferative response to peptide 49 - 63 was not due to a contamination, or an error arising during its synthesis, peptide from two suppliers was examined. Peptide from Altabioscience, Birmingham was used as the major source of antigen for this study and was compared to peptide supplied by Genesis, Cambridgeshire (Figure 6.14). Almost identical responses to the two peptides were seen in the two healthy individuals (JF and JM) and two patients with SLE (HJ and BF). Responses were fractionally higher to the peptide supplied by Genesis. This was possibly due to its higher purity. Peptides supplied by Altabioscience were not HPLC purified while those from Genesis were.

6.5.2 Is the proliferative response to peptide 49 - 63 a naive or primed T cell response?

A proliferative response to peptide 49 - 63 was seen in both healthy individuals and patients with the HLA DR3 DQ2 haplotype using unfractionated PBMC's. However, the assay system fails to distinguish the nature of the response. Cell populations were therefore isolated in order to determine if the response was mediated by either naive (CD45RA) or primed (CD45RO) CD4 T cells. CD45RA and CD45RO T cells were isolated from peripheral blood and cultured with autologous antigen presenting cells, anti - CD28 monoclonal antibody and peptide 49 - 63 for 7 days. Mean proliferative responses equal to or greater than a stimulation index of three were considered as positive. Three healthy

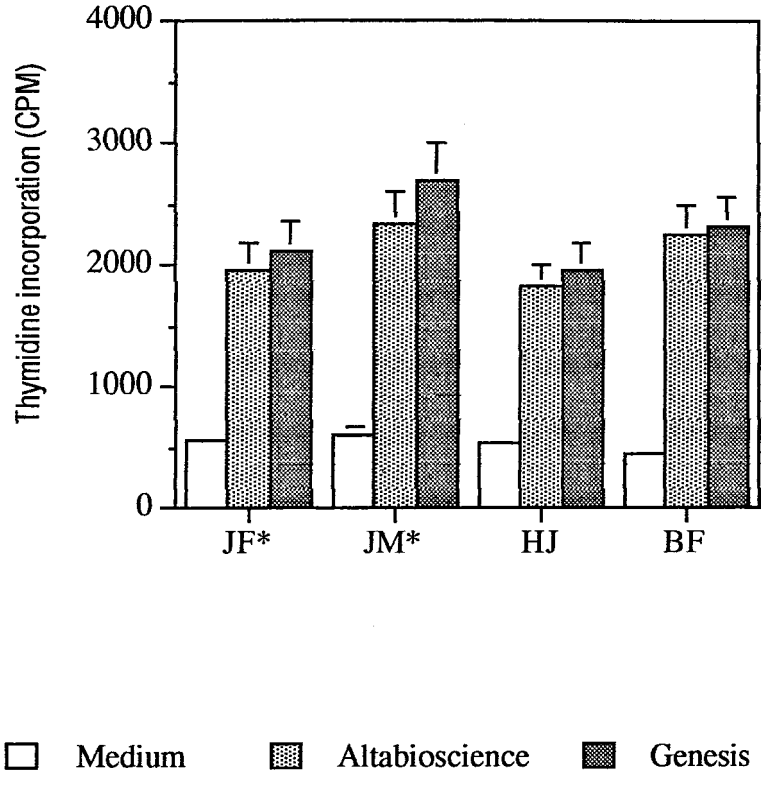


Figure 6.14

Proliferative response (tritiated thymidine incorporation) in two healthy individuals (JF and JM) and two patients (HJ and BF) to peptide 49 - 63. The peptide was obtained from two suppliers; Altabioscience, Birmingham and Genesis, Cambridgeshire. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation. * denotes male subjects.

individuals (JM, JF and PB) and three patients with SLE (HJ, JC and BF) were studied. Using unfractionated PBMC's all of the subjects responded to peptide 49 - 63. Purified T cells were analysed by flow cytometry (Figures 6.15, 6.16 and Table 6.4). The preparations contained less than 4% contamination with CD8, CD14, CD16 or CD19 except for patient HJ. CD45RA purified cells were greater than 95% pure while CR45RO purified cells were greater than 97% pure except for HJ (92% CR45RA and 84% CD45RO CD4 T cells).

The response to PHA, tetanus toxoid, PPD and rabies protein was examined in both T cell populations to confirm that the purification had been accomplished successfully and to demonstrate a naive and primed response. CD45RA (naive) and CD45RO (primed) T cells from healthy individuals (Figure 6.17) and patients with SLE (Figure 6.18) responded to PHA. In the healthy individuals the response to tetanus toxoid and PPD occurred predominantly in the primed T cell population but to a much less extent in the naive population (Figure 6.17). Individual PB was not tested for a response to PPD due to insufficient yield from the purification steps. The response to rabies protein occurred in the naive T cell population only indicating CD45RA T cells had been successfully purified.

Patients BF and JC showed a poor proliferative response to tetanus toxoid using unfractionated PBMC's and failed to show a response in the CD45RA and CD45RO T cell fractions to this antigen. However, patient BF and JC responded to PPD using unfractionated PBMC's (Figure 6.1 and Appendix A). Neither patient responded to PPD in the CD45RA T cell population. Patient JC, but not BF, showed a good proliferative response to PPD in the CD45RO T cell population. Patients BF and JC also failed to respond to rabies protein in either of the T cell populations. Patient HJ responded to tetanus toxoid, PPD and rabies protein with a SI ≥ 3 using unfractionated PBMC's (Figure 6.1 and Appendix A). However, using fractionated T cells a proliferative response was seen only when stimulating CD45RO T cells with tetanus toxoid. A response to PPD was not tested due to an insufficient cell yield.

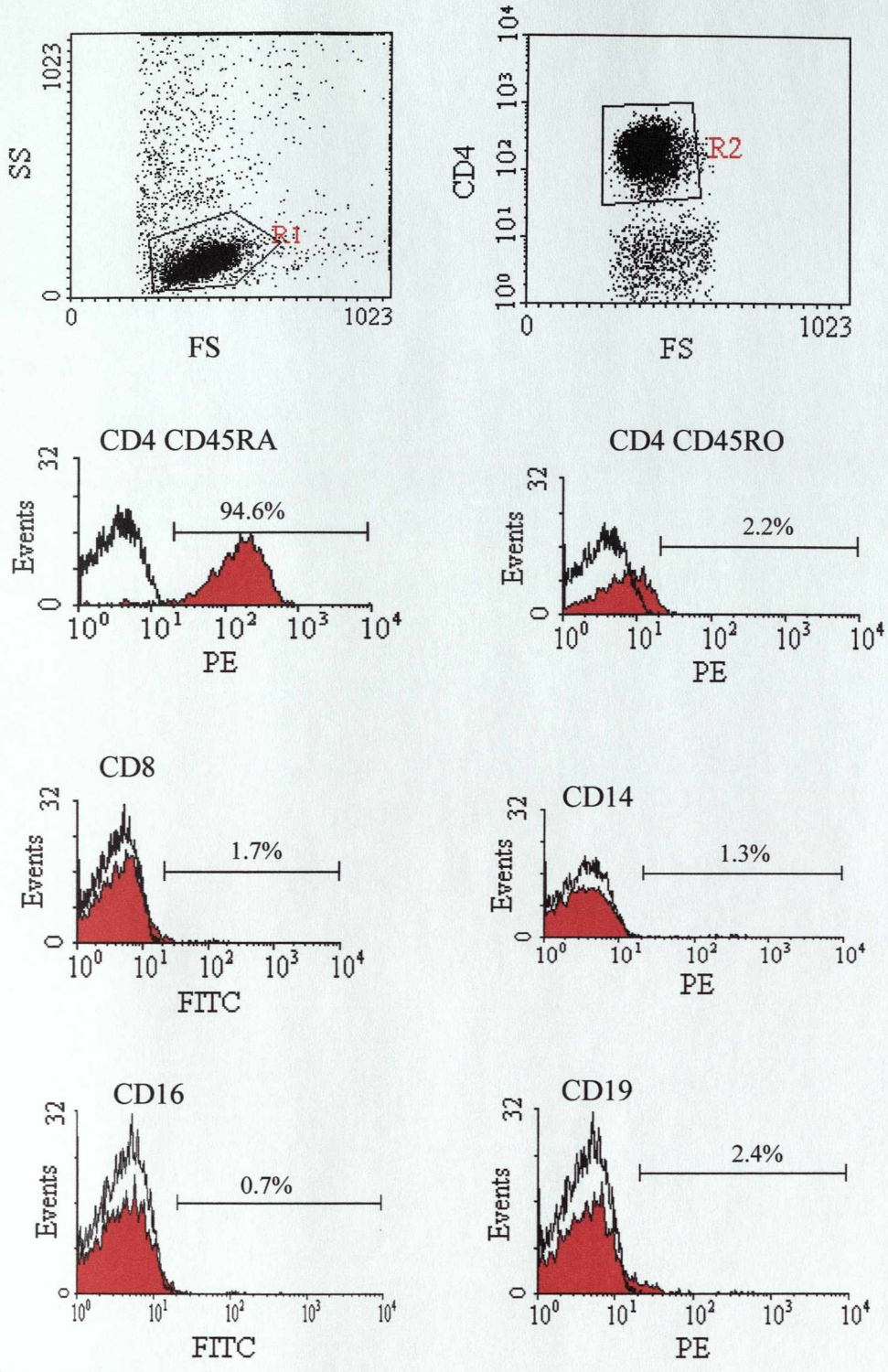


Figure 6.15

FACS analysis of purified CD4 CD45RA T cells from patient JC. CD4 T cells were gated to determine the percentage of CD45RA and CD45RO cells. Cells were ungated to determine the percentage of CD8, CD14, CD16 and CD19.

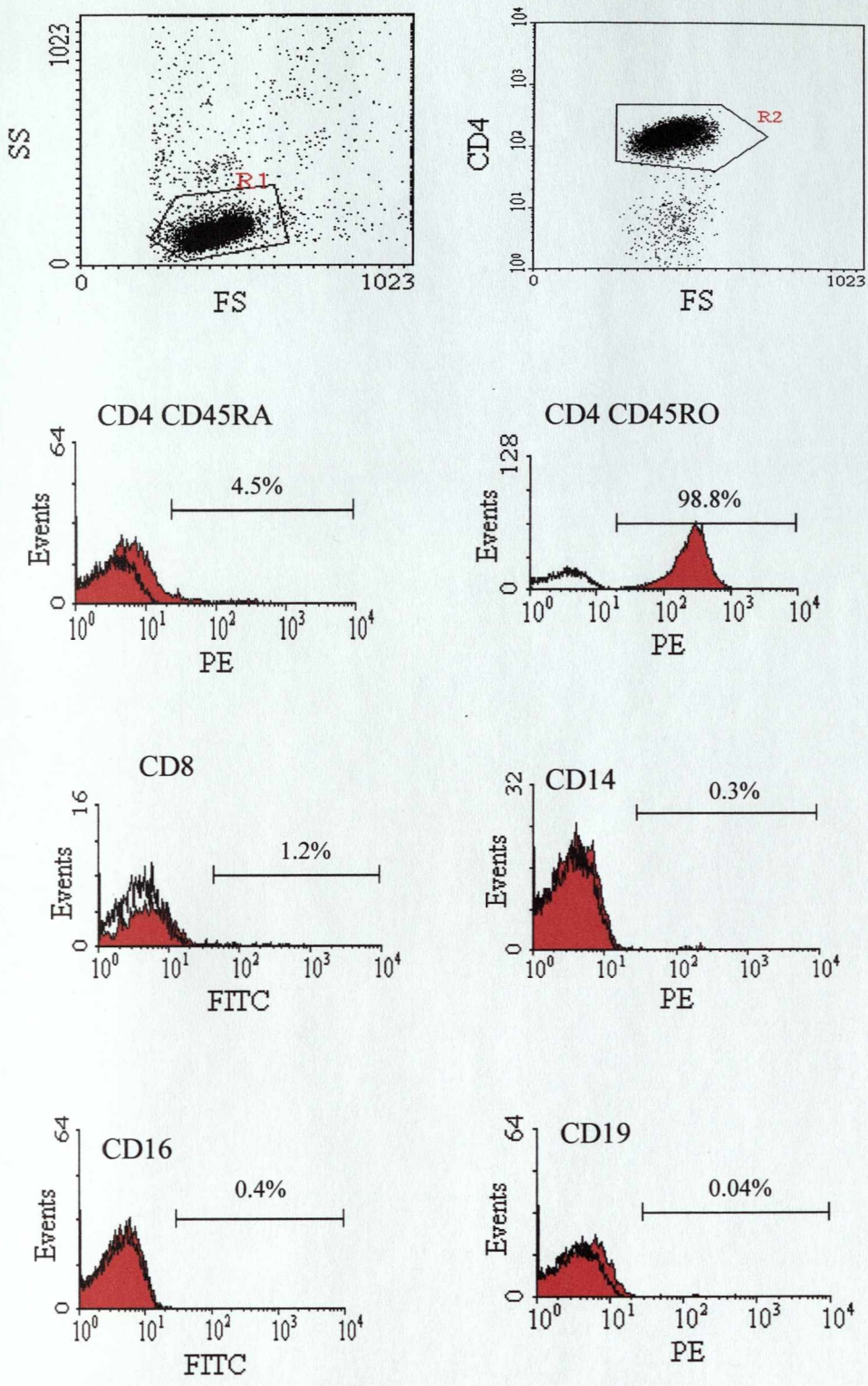


Figure 6.16

FACS analysis of purified CD4 CD45RO T cells from patient JC. CD4 T cells were gated to determine the percentage of CD45RA and CD45RO cells. Cells were ungated to determine the percentage of CD8, CD14, CD16 and CD19.

CD4 CD45RA purified T cells

	*CD4 CD45RA	*CD4 CD45RO	#CD8	#CD14	#CD16	#CD19
Healthy individuals						
JF	95.8	8.6	1.6	2.5	1.7	2.4
JM	98.3	5.8	4.5	0.4	0.6	0.4
PB	99.2	14.6	2.5	2.9	2.4	0.5
Patients						
HJ	92.1	5.8	11.6	1.3	3.8	9.6
JC	94.6	2.2	1.7	1.3	0.7	2.4
BF	99.1	1.7	0.9	0.2	0.2	2.3

CD4 CD45RO purified T cells

	*CD4 CD45RA	*CD4 CD45RO	#CD8	#CD14	#CD16	#CD19
Healthy individuals						
JF	21.9	98.5	2.2	0.4	0.5	0.4
JM	10.5	97.2	2.2	0.7	1.5	0.6
PB	10.9	98.4	0.3	1.8	0.7	0.6
Patients						
HJ	25.9	84.2	5.8	1.8	2.7	2.2
JC	4.5	98.8	1.2	0.3	0.4	0.04
BF	10	98.2	0.4	0.1	0.3	0.1

Table 6.4

Expression of cell surface markers as determined by flow cytometry of CD4 CD45RA and CD4 CD45RO purified T cells as a percentage of either CD4 gated (*) or ungated (#) cells. Naive and primed T cells were purified in three healthy individuals (JF, JM and PB) and three patients (HJ, JC and BF) with the HLA DR3 DQ2 haplotype.

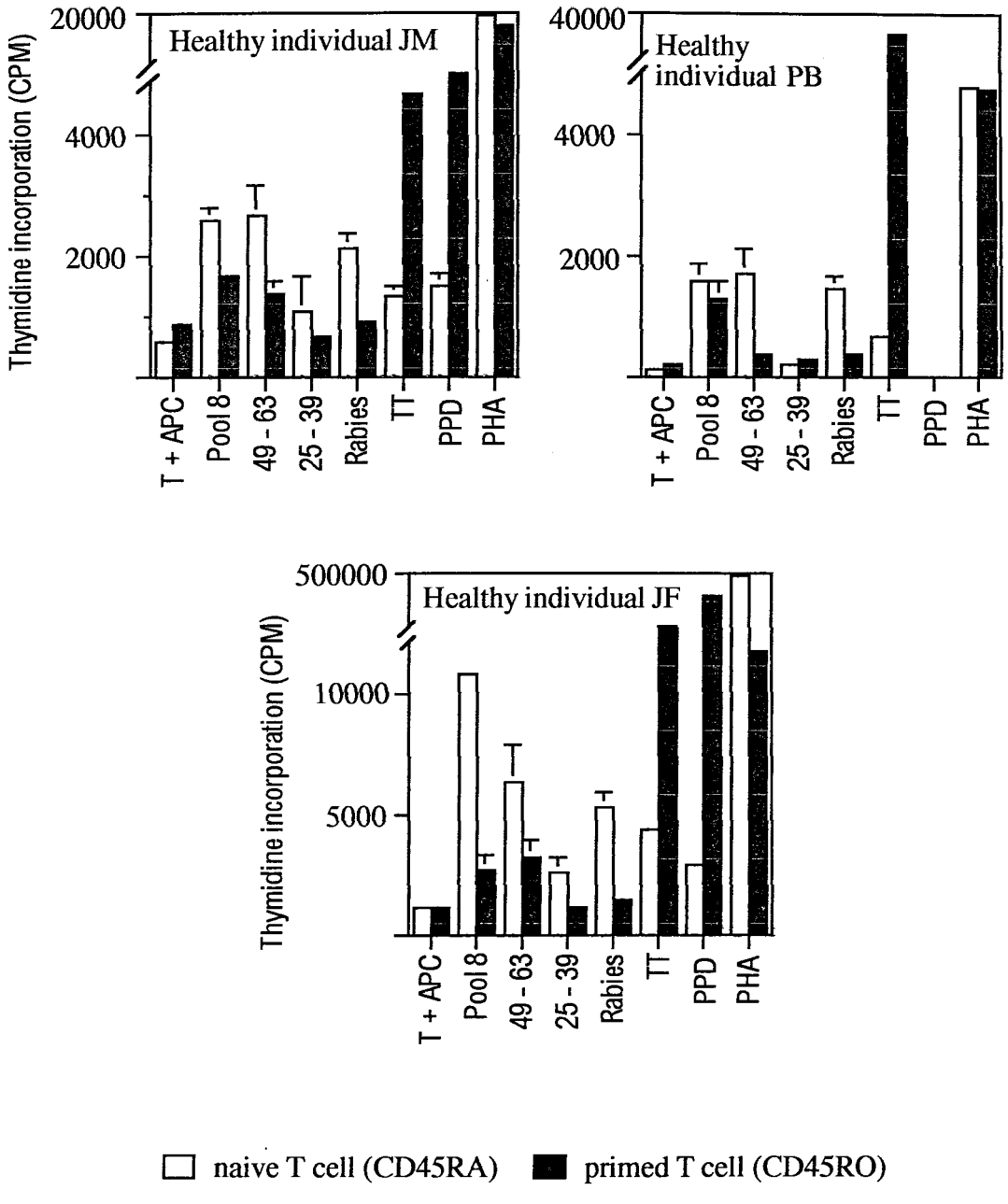


Figure 6.17

Proliferative response in the naive and primed T cell populations to peptide pool 8, La peptides 49 - 63 and 25 - 39, rabies protein, tetanus toxoid, PPD and PHA in three healthy individuals with the HLA DR3 DQ2 haplotype. The results are expressed as mean CPM of the replicate wells ± standard deviation.

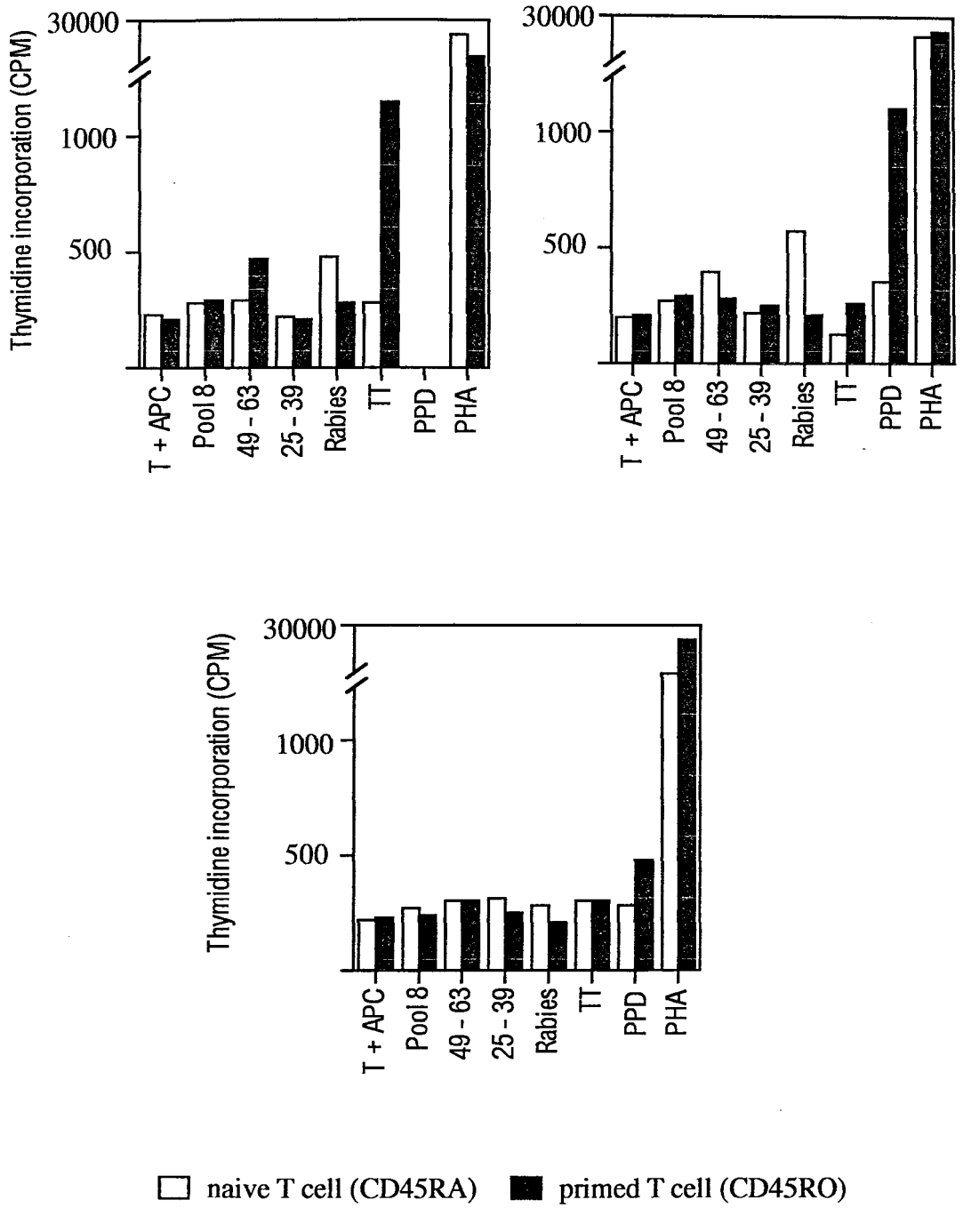


Figure 6.18

Proliferative response in the naive and primed T cell populations to peptide pool 8, La peptides 49 - 63 and 25 - 39, rabies protein, tetanus toxoid, PPD and PHA in three patients with the HLA DR3 DQ2 haplotype. The results are expressed as mean CPM of the replicate wells ± standard deviation.

The response to peptide pool 8, peptide 49 - 63 and a control La peptide 25 - 39 was examined in the CD45RA and CD45RO T cell populations in the six subjects. Using unfractionated PBMC's all six subjects responded to peptide 49 - 63 (Table 6.3) All three healthy individuals responded, with a SI ≥ 3, to pool 8, peptide 49 - 63 and rabies protein in the CD45RA T cell population (Figure 6.17). Additionally, individual PB responded to peptide pool 8 in the CD45RO T cell population. The three patients failed to respond to peptide pool 8 and peptide 49 - 63 in either the CD45RA or CD45RO T cell populations with a SI ≥ 3 (Figure 6.18). However, patient HJ responded to rabies protein in the CD45RA T cell population and peptide 49 - 63 in the CD45RO T cell population with a SI ≥ 2. Patients JC and BF failed to respond to the La peptides with a SI ≥ 2. Repeat experiments did not show improved proliferative responses to the antigens tested in either the naive or primed T cell populations. Peptide 25 - 39 did not stimulate a response in either T cell population in healthy individuals or patients.

6.5.3 Is the response to peptide 49 - 63 a HLA restricted response?

The HLA DR3 DQ2 haplotype is associated with an increased risk of SLE and presence of serum anti - La antibodies. To examine the role of the HLA DR and HLA DQ molecules in the proliferative response to peptide 49 - 63 (amino acid sequence LEIMIKFNRLNRLTT) individuals possessing HLA DR3 DQ2, HLA DQ2, HLA DQ6 or neither were examined. Individuals expressing HLA DR3 without HLA DQ2 were not available for this study. Individuals expressing HLA DR1 were selected as a control population. Unfractionated PBMC's from the subjects were cultured with the peptide and anti - CD28 antibody for 7 days. Four of six healthy individuals and four of nine patients with the HLA DR3 DQ2 haplotype showed a proliferative response to peptide 49 - 63 (Table 6.3). Healthy individuals with the HLA DQ2 or DQ6 haplotype failed to respond to the peptide although they responded to peptide pools 1 and 8. These data therefore suggest that the proliferative response to peptide 49 - 63 is restricted by the HLA DR molecule although it appears that the HLA DQ molecule is required for responses to other La peptides. Healthy individuals with the HLA DR1 haplotype and patients with the HLA DR4 haplotype failed to

responded to peptide 49 - 63 (Table 6.3). However, healthy individuals with the HLA DR1 haplotype responded to pool 1 suggesting that the HLA DR4 molecule is protective of responses to La peptides.

MHC restriction was also examined using antibodies to the HLA DR molecule. Anti - HLA DR antibody (L243 culture supernatant) was added to cultures containing PBMC's and peptide 49 - 63. An antibody to HLA DQ was not available at the time of study. Preliminary data showed that anti - HLA DR antibody inhibited the response by peptide 49 - 63 in a dose dependent manner (Figure 6.19). A $^1/_{10}$ dilution of the antibody inhibited the response to the peptide completely. However, it cannot be excluded that the response was due to toxicity of the antibody since an isotype matched control antibody was not included.

6.5.4 Summary of the proliferative response to peptide 49 - 63

In summary, to confirm that the proliferative response to peptide 49 - 63 was not due to contamination or an error during peptide synthesis, peptide was obtained from a second supplier. Peptides from both suppliers stimulated similar proliferative responses (Figure 6.14). The proliferative response was examined further in CD45RA and CD45RO T cell populations. Healthy individuals responded to peptide pool 8 and peptide 49 - 63 in the CD45RA T cell population with a SI $\geq$ 3 (Figure 6.17). As control antigens for cell purity the response to rabies protein was found in the CD45RA T cell population and the response to recall antigens, tetanus toxoid and PPD, were predominantly found in the CD45RO T cell population. Patients failed to respond to either peptide pool 8 or peptide 49 - 63 in either T cell population with a SI $\geq$ 3 (Figure 6.18). They also failed to respond to rabies protein although two of the three patients responded to a recall antigen in the CD45RO T cell population with a SI $\geq$ 3. However, patient HJ responded to peptide 49 - 63 in the CD45RO T cell population and rabies protein in the CD45RA T cell population with a SI $\geq$ 2. The poor proliferative responses in fractionated T cells from patients with SLE therefore prevents a clear conclusion, regarding which of the T cell populations is responding to peptide 49 - 63, to be drawn.

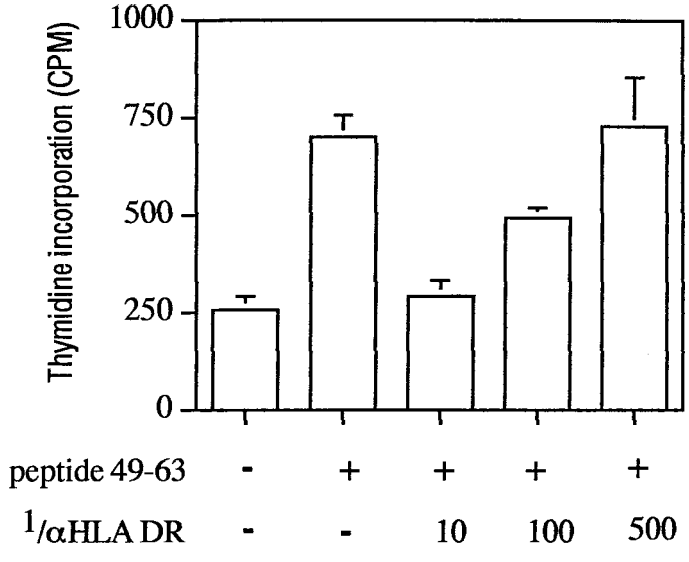


Figure 6.19

Proliferative response to the La peptide 49 - 63 with or without the addition of an anti - HLA DR antibody (L243). Proliferation assays were carried out as described in section 3.3.9 using PBMC's from a healthy individual with the HLA DR3 DQ2 haplotype. The results are expressed as mean CPM of the replicate wells ± standard deviation.

Experiments using PBMC's from subjects with either the HLA DR3 DQ2, HLA DR1 or HLA DQ2 or DQ6 haplotype suggest that the proliferative response to peptide 49 - 63 is mediated by HLA DR molecules (Table 6.3). Preliminary data using anti - HLA DR antibodies confirms this finding (Figure 6.19). However, the HLA DQ molecule appears necessary for proliferative responses to the peptide pools (see section 6.3).

6.6 Human native La

A stimulatory epitope of the La protein, peptide 49 - 63, has been identified but it is unknown if this peptide represents a naturally processed and presented epitope or a cryptic epitope. This is an essential question which requires answering so preliminary work was carried out into the feasibility of extending the project in this direction. The aim of further research would involve determining if T cell lines specific to the human La protein could respond to the La peptide 49 - 63. Initial experiments were carried out to determine if sufficient human La protein could be purified from human cells and to examine the proliferative responses to the purified native protein.

6.6.1 Purification of native La protein

Human native La is not available commercially so attempts were made to purify it from human cells. U937, HeLa, MOLT-4 and K562 cell lines were examined for the expression of La protein (Figure 6.20). HeLa cells and K562 cells were found to be good sources of human La protein. However, K562 cells were used since HeLa cells are adherent and are more difficult to manage in the tissue culture facilities available. The La protein was purified by ion exchange chromatography and heparin affinity chromatography (see section 3.4). La was eluted from the ion exchange chromatography column with buffer C containing 500mM NaCl. After dialysis this fraction was loaded on to the heparin affinity column and the La protein was eluted with buffer D containing 1M NaCl (Figures 6.21 and 6.22). La protein was also present in the flow through fraction of the heparin column. However, the buffer D fraction was used after dialysis into PBS as the source of La protein

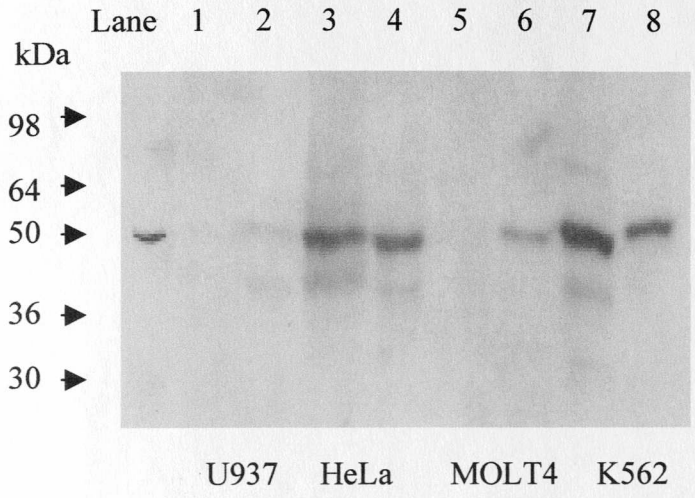
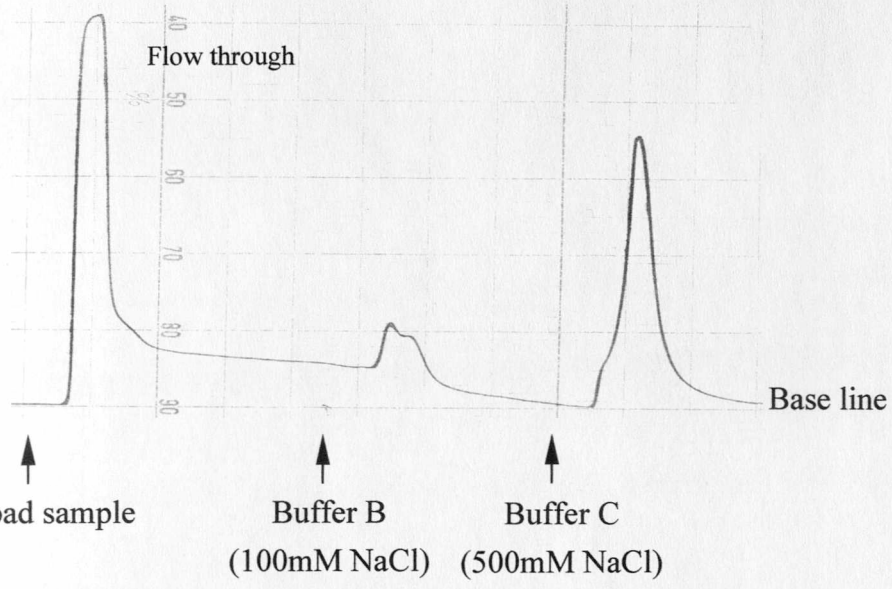


Figure 6.20
U937 (lane 1 and 2), HeLa (lane 3 and 4), MOLT4 (lane 5 and 6) and K562 (lane 7 and 8) cell lines were tested by Western blot for the presence of La protein. The supernatant (lane 1, 3, 5 and 7) and cytoplasmic (lane 2, 4, 6 and 8) levels were compared. Proteins were loaded at 10μg per lane. Western blots were probed with the anti-La antibody SW5 (see sections 3.4.3 and 3.4.4).

DEAE ion exchange chromatography



Heparin affinity chromatography

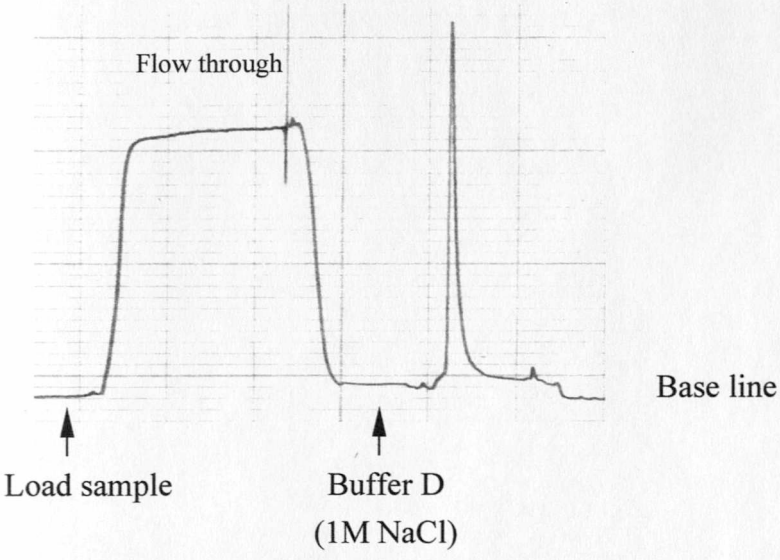


Figure 6.21
Typical traces obtained from DEAE ion exchange chromatography and heparin affinity chromatography (see section 3.4.2).

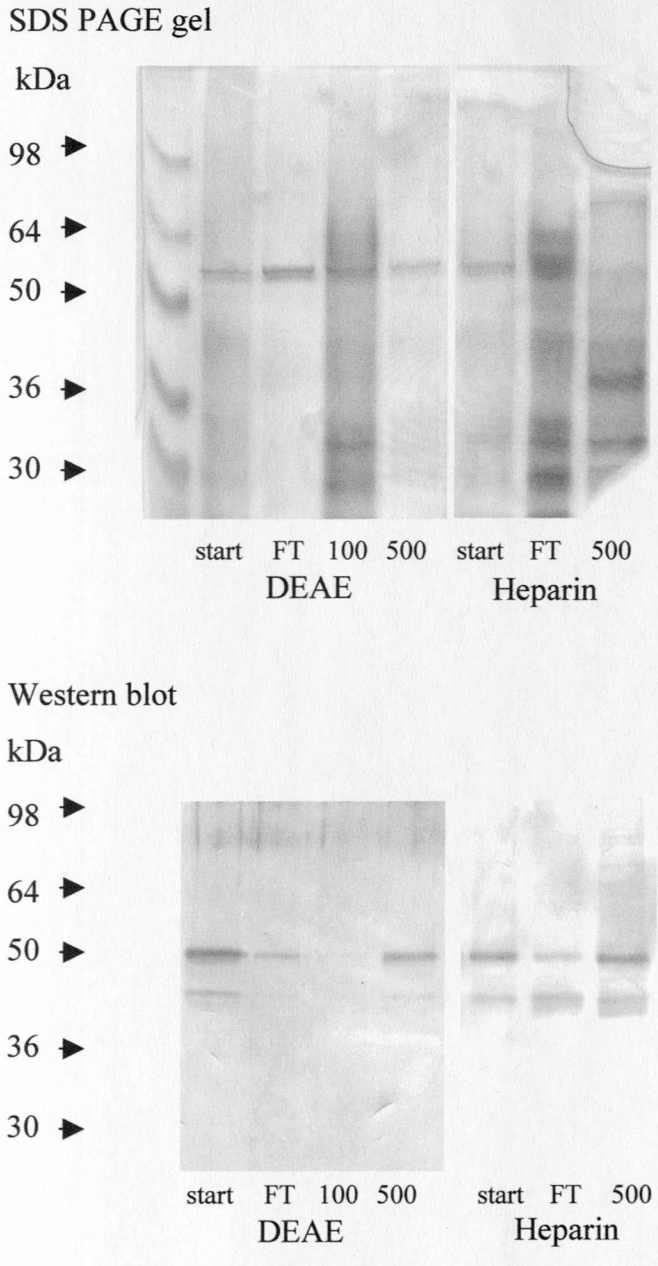


Figure 6.22
La protein was purified from K562 cell lysate using DEAE ion exchange chromatography and heparin affinity chromatography. Eluted fractions were tested by SDS PAGE (see section 3.4.3) or by Western blot probing the membrane the with anti-La antibody SW5 (see section 3.4.4). Protein was loaded at 10µg per lane.

to examine the proliferative response since it was cleaner than the La present in the flow through (Figure 6.23). Additionally, according to Stefano (1984), the La protein eluted with buffer D should be free of RNA. Figure 6.23 shows a SDS PAGE gel and a Western blot of the final partially purified La protein. The contaminating proteins could not be identified by Western blotting.

6.6.2 Proliferative response to partially purified La protein

The proliferative response to the partially purified La protein was examined in six healthy individuals and nine patients with the HLA DR3 DQ2 haplotype, six healthy individuals and three patients with the HLA DR1 or DR4 haplotype and six healthy individuals with the HLA DQ2 or DQ6 haplotype. Fresh PBMC's were cultured for 7 days with the La protein preparation and anti - CD28 antibody (see section 3.3.9). The flow through fraction from the heparin column failed to stimulate a proliferative response. The optimal proliferative response occurred using 10μg/ml of partially purified protein (Figure 6.24). The La preparation was not considered toxic to the cells as it did not inhibit the proliferative response to tetanus toxoid (Figure 6.25). Two of the 6 healthy individuals and 5 of the 9 patients with the HLA DR3 DQ2 haplotype showed a proliferative response to the partially purified La protein with a SI $\geq$ 3 (Figure 6.26 and Table 6.3). The two healthy individuals (JF and DP) responding to the purified La failed to respond to pools 1 or 8 although JF responded to peptide 49 - 63. The five patients responding to the purified La also responded to peptide pool 1 or 8 while four of the five patients responded to peptide 49 - 63. None of the individuals with the HLA DR1 or DR4 haplotype responded to the partially purified La. Four of the 6 healthy individuals with the HLA DQ2 or DQ6 haplotype responded to the partially purified La of which three responded to peptide pool 1 and 8 (Table 6.3).

6.6.3 Summary of the proliferative responses to human native La

In summary, proliferative responses were identified to the La peptide 49 - 63. However it

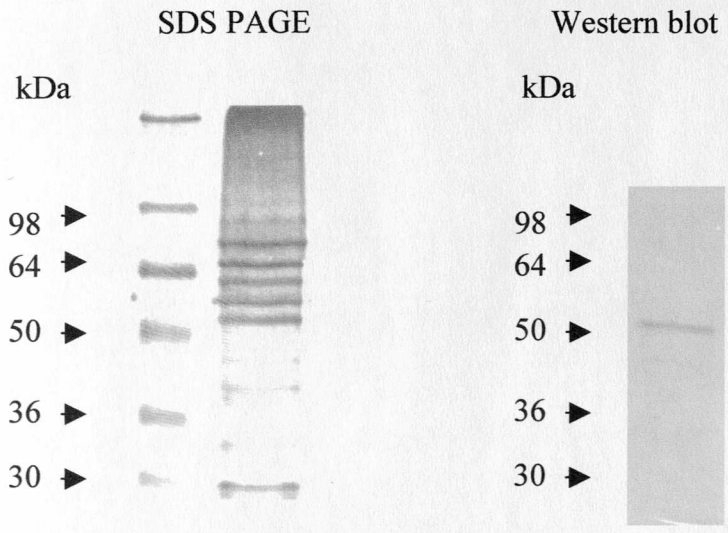


Figure 6.23
Partially purified human La preparation obtained using DEAE ion exchange column chromatography and heparin affinity chromatography. Protein was loaded at 10μg per lane. Western blots were probed with the anti-La antibody SW5 (see sections 3.4.3 and 3.4.4).

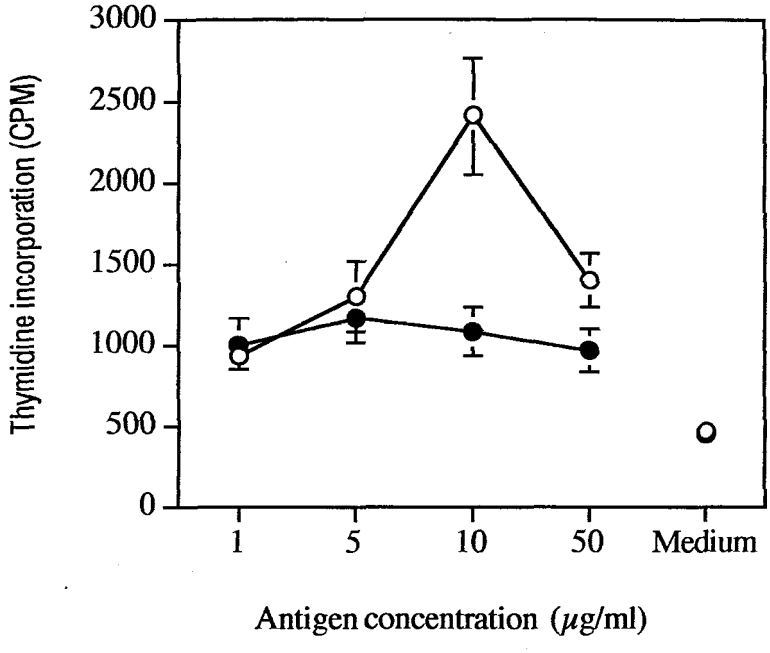


Figure 6.24

Proliferative response (tritiated thymidine incorporation) in a healthy individual to the heparin flow through fraction or the partially purified La protein. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation.

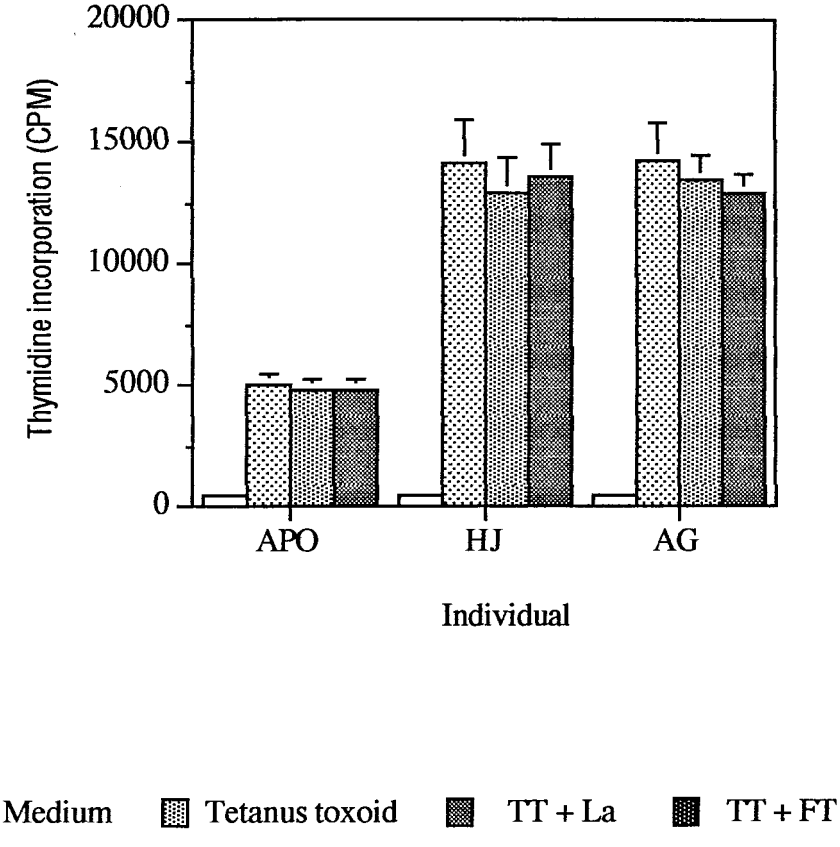


Figure 6.25

Proliferative response (tritiated thymidine incorporation) to tetanus toxoid (TT) alone, with partially purified La protein (TT + La) or heparin flow through fraction (TT + FT). Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of the replicate wells ± standard deviation.

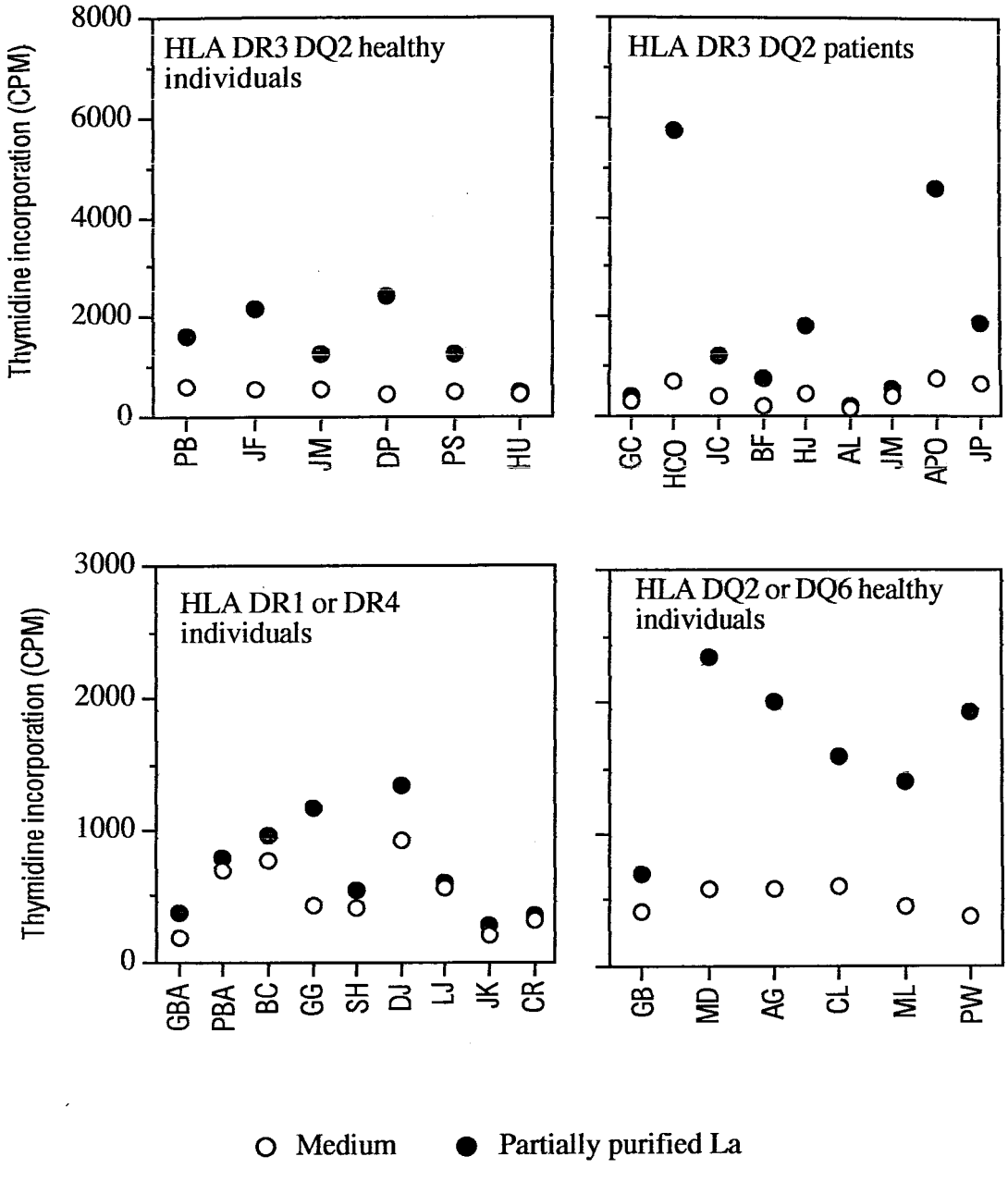


Figure 6.26

Proliferative response (tritiated thymidine incorporation) to medium or partially purified La protein in healthy individuals and patients with the HLA DR3 DQ2, HLA DR1 or DR4, or HLA DQ2 or DQ6 haplotype. Proliferation assays were carried out as described in section 3.3.9. The results are expressed as mean CPM of triplicate wells.

is unknown if this peptide represents a naturally processed epitope or a cryptic epitope. To show that peptide 49 - 63 is a naturally processed antigen the ability of the peptide to stimulate T cell lines specific for whole La would have be to demonstrated. Healthy individuals and patients with the HLA DR3 DQ2 haplotype and healthy individuals with the HLA DQ2 or DQ6 haplotype showed a proliferative response to the partially purified La protein with a SI $\geq$ 3. Patients with the HLA DR3 DQ2 haplotype who responded to peptide 49 - 63 also responded to whole La (Table 6.3). Subjects with the HLA DR1 or DR4 haplotype failed to respond to the La protein. It cannot be excluded, however, that the proliferative response to the partially purified La protein is to contaminating proteins. To clarify this issue the La protein would require further purification.

6.7 Discussion

The La protein is predicted to be a T cell autoantigen in SLE as serum anti - La antibodies are found in patients with this disease. The antibodies are IgG class switched and show somatic hypermutations further suggesting that there is an underlying T cell mechanism. Furthermore, there is a close association between the presence of anti - La antibodies and the possession of the HLA DR3 DQ2 haplotype. Helsloot and Sturgess (1997) and Halse *et al.* (1996) identified T cells recognising recombinant La in peripheral blood of healthy individuals and patients with SLE or SS. However, there was no difference in the number of healthy individuals or patients responding to the protein. The T cell responses were not characterised or the stimulatory epitopes identified. However, Helsloot and Sturgess (1997) attempted a limiting dilution analysis but identified abnormally low precursor cell frequencies for tetanus toxoid. The recombinant preparations were not tested for bacterial antigen which may be responded to instead of the antigen of interest (Hawke *et al.*, 1996; Life *et al.*, 1991; Matsuo *et al.*, 1996).

As La specific T cells to whole antigen were not identified specifically in patients it is hypothesised that the La protein is modified prior to its presentation to the T cell. The number of possible modifications is endless and include glycosylation, phosphorylation,

deamidation and specific cleavage. In the present study T cell responses to synthetic peptides representing the entire La protein were examined in order to identify epitopes revealed after specific cleavage. Peptides of 15 amino acid residues were used since peptides of this length are more efficient at identifying dominant T cell epitopes (Reece *et al.*, 1994). PBMC's taken from healthy individuals and patients with SLE were used since they represent a T cell repertoire which has not been biased by *in vitro* selection.

6.7.1 Subjects and HLA haplotype

Subjects were selected on the basis of their HLA haplotype and the presence of serum anti - La antibodies. HLA DR2, DR3, DQ2 and DQ6 have been reported to be associated with serum anti - La antibodies in patients with SLE (Vyse and Kotzin, 1998). The HLA DR3 is in strong linkage disequilibrium with HLA DQβ1*0201. HLA DR1 and HLA DR4 have not been associated with anti - La antibodies in any study. Patients selected for the present study conformed to the published reports with respect to serum anti - La antibody titres and HLA haplotype.

6.7.2 La peptide pools covering residues 1 - 72 and 121 - 192 are stimulatory

Proliferative responses to the 15mer La peptides covering the entire La sequence were examined in PBMC's taken from selected individuals. Since the peripheral blood was expected to exhibit a low frequency of T cells specific for the peptides all assays were carried out using a minimum of five replicate wells per test group. Using a pooling strategy similar to Lohmann *et al.* (1996) for identifying T cell epitopes to glutamic acid decarboxylase (GAD), two regions of the La protein were identified as stimulatory; region 1 - 72 (pools 1 and 8) and region 121 - 192 (pools 3 and 10). However, using this pooling strategy it cannot be excluded that individual peptides may be missed if some peptides within the pool can bind to the MHC but do not contain a T cell epitope. These MHC binding peptides therefore may inhibit the response by stimulatory peptides and prevent them from being measured. Proliferative responses were identified in healthy individuals

and patients with the HLA DR3 DQ2 haplotype and healthy individuals with the HLA DQ2 or DQ6 haplotype. There was no difference in the stimulation index values between healthy individuals and patients. Proliferative responses to autoantigens have previously been described in healthy individuals including responses to GAD (Lohmann *et al.*, 1996), ribosomal P2 protein (Crow *et al.*, 1994), gliadin (O'Keeffe *et al.*, 1999) and topoisomerase I (Kuwana *et al.*, 1995). It was therefore possible that healthy individuals in this study would respond to the La peptides. This is possibly due to a lack of T cell tolerance *in vivo*.

Four of seven healthy individuals and five of six patients with the HLA DR1 or HLA DR4 haplotype failed to respond to any of the peptide pools. There was no obvious explanation as to why some of these individuals responded to the peptide pools since the haplotype is not associated with anti - La antibodies.

The mean tritiated thymidine incorporation values for replicate wells stimulated with the peptide pools was low and the standard deviations were large. This indicated that proliferative responses did not occur in all of the replicate wells. This was probably as a consequence of the low number of La specific T cells present in peripheral blood. Each individual well was therefore assigned as a positive or negative using a stimulation index of three for the unstimulated wells as the cut off point. This analysis revealed that the proliferative response to amino acid regions 1 - 72 and 121 - 192 occurred in more of the replicate wells in patients compared to healthy individuals both with the HLA DR3 DQ2 haplotype. Additionally, for peptide pools covering these regions, the number of responding wells in HLA DR3 negative but HLA DQ2 or DQ6 positive healthy individuals was similar to that in HLA DR3 DQ2 patients. Since approximately 80% of replicate wells responded to tetanus toxoid (representative or a primed response) but only 30 - 50% responded to rabies protein (representative of a naive response) it was suggested that the response in HLA DQ2 subjects is mediated by the primed T cells and the response in HLA DR3 subjects is mediated by naive T cells. In the HLA DR3 healthy individuals the response to the peptide pools was equivalent to the response to the Hsp65 control

peptide (representative of a primed response) in terms of the stimulation index. However, a fewer number of replicate wells responded to the peptide pools compared with the Hsp65 peptide. This further suggests that the response in HLA DR3 subjects is mediated by the naive T cell population. However, the number of peptide pools to which the patients respond suggests that HLA DR and HLA DQ molecules play a role in the response to La protein.

6.7.3 Peptide 49 - 63 is the dominant stimulatory peptide

Forty individual peptides covering regions 1 - 72 and 121 - 192 were examined for the ability to stimulate a proliferative response in PBMC's from selected individuals. Five peptides were identified which stimulated a mean proliferative response of SI $\geq$ 3, although peptide 49 - 63 was clearly more stimulatory in more individuals (Figure 6.12). A stimulatory peptide was not identified within region 121 - 192 when the peptides were tested individually although a proliferative response was measured to a pool of ten peptides covering this region. This suggests that multiple epitopes are present and that all peptides are required to stimulate a proliferative response with a SI $\geq$ 3.

Proliferative responses to peptide 49 - 63 were found only in healthy individuals and patients with the HLA DR3 DQ2 haplotype. This suggests that peptide 49 - 63 is presented by the HLA DR molecule. Reynolds *et al.* (1996) showed that the dominant T cell epitopes in T cells isolated from mice immunised with human La covered residues 61 - 84 and 288 - 302. Subdominant epitopes covered residues 13 - 30, 25 - 44 and 106 - 129. Although similar sequences were not identified in the two systems murine and human La sequences are only 77% identical. Additionally, some epitopes may have been missed using the current peptides since the 15mer peptides may not be the correct length or epitopes were contained within the three amino acid overlap between consecutive peptides. An overlap of one amino acid is ideal but increases the number of peptides to study.

The MHC restriction was confirmed using an anti - HLA DR antibody (L243) which

completely inhibited the proliferative response to the peptide in healthy individual JF (homozygous for the HLA DR3 haplotype). However, an isotype control antibody was not included in the assay for comparison. Peptide 49 - 63 was resynthesised to confirm that the response was not due to contaminants or an error during synthesis; including mini deletions, insertions or persisting protecting groups which can go undetected. Proliferation was very similar in response to the peptides obtained from two separate sources. However, Nagvekar *et al.* (1999) report that T cell responses to a synthetic acetylcholine receptor peptide were directed to a recurring error in the peptide obtained during its synthesis.

6.7.4 MHC restriction

The proliferative response to peptide 49 - 63 mirrors the possession of the HLA DR3 haplotype but not the presence of serum anti - La antibodies. Since individuals with the HLA DR3 DQ2 or HLA DQ2 haplotype responded to the peptide pools but only individuals with the HLA DR3 DQ2 haplotype responded to peptide 49 - 63 the role of both the HLA DR and HLA DQ molecules in the response to La cannot be excluded. It is difficult to evaluate the role of each of the MHC molecules since HLA DR3 and HLA DQ2 are in strong linkage disequilibrium. However, Reveille *et al.* (1991) reported that anti - Ro and anti - La antibodies are more strongly associated with the HLA DQβ1* allele, having a leucine at position 26 at the outer most domain of the molecule. This residue maps to the floor of the class II molecule and therefore could play a preferential role in the presentation of peptides. If the antibody response is mediated via the HLA DQ molecule it could be speculated that the T cell response is mediated via the HLA DR molecule. However, HLA DP molecules may also play a role although it is unclear if variation at this locus is associated with an increased risk of SLE (Davies *et al.*, 1994; Fronek *et al.*, 1997).

Interestingly, peptide 49 - 63 is recognised equally well by healthy individuals and patients with the HLA DR3 DQ2 haplotype, although patients respond to a broader range of peptides. The proliferative response to peptide 49 - 63 indicates that the peptide contains both a MHC binding site and a T cell epitope. However, it can not be excluded that peptide

49 - 63 is capable of binding non - specifically to MHC molecules. The peptide may aggregate and increase the chances of being engulfed by APC's (Wu *et al.*, 1993). Farris *et al.* (1999) showed that multimeric dominant and subdominant La peptides, but not monomeric La peptides could induce anti - La antibodies in mice. It is possible that the monomeric forms do not provoke adequate T cell help but that the multimeric form initiates the disease which is maintained by the monomeric form. Additionally, synthetic peptides are prone to oxidation leading to damaged epitopes or an altered ability to bind MHC and can adopt unnatural conformations after binding directly to surface MHC (Viner *et al.*, 1996). Hence, the peptide 49 - 63 may be not be relevant in the disease process of SLE.

In contrast to the present study in which healthy individuals and patients respond to the same peptide i.e. peptide 49 - 63, Lohmann *et al.* (1996) showed that T cell epitopes to glutamic acid decarboxylase differ in healthy individuals and patients. In the present study patients with SS were not examined for their ability to response to individual peptides but it would be interesting to determine if they responded to different epitopes compared to patients with SLE. Tzioufas *et al.* (1997) showed that the B cell epitope differs between patients with SLE and SS. Differences in T cell epitopes, as well as B cell epitopes, may help to explain the immunological mechanisms and the differences in the manifestations of the two diseases.

A number of B cell epitopes to La protein have been identified. The dominant epitope covers amino acids 111 - 242 of the La protein although epitopes are spread throughout the protein (Weng *et al.*, 1993; Rauh *et al.*, 1988; Sturgess *et al.*, 1988; Topfer *et al.*, 1995). Rischmueller *et al.* (1995) showed that epitope 111 - 242 is a conformational epitope which associates with the Ro60 protein. It may therefore be speculated that anti - La antibodies compete with Ro60 for the binding to the B cell epitope 111 - 242. Potentially, both the anti - La antibody and Ro protein could bind to the La protein if appropriate conformational changes occurred in the La protein. The conformational changes may reveal epitopes which are not normally available to the immune system, including epitope /

peptide 49 - 63. This hypothesis may only partially explain the mechanism involved in SLE since PBMC's taken from healthy individuals also proliferate in response to stimulation with peptide 49 - 63. However, Venables *et al.* (1988) showed that anti - La antibodies could be produced *in vitro* in healthy individuals with the HLA DR3 haplotype following stimulation of PBMC's with PWM. This implies that the HLA DR3 molecule may be important in the disease process.

6.7.5 Predicted MHC - peptide interaction

Peptide 49 - 63 is stimulatory in healthy individuals and patients with the HLA DR3 DQ2 haplotype but not in healthy individuals with the HLA DQ2 or DQ6 haplotype suggesting that the HLA DR3 molecule is responsible for presenting this peptide to T cells. Additionally, anti - HLA DR antibody was shown in the present study to prevent a proliferative response to the peptide in a homozygous HLA DR3 healthy individual. The HLA typing did not identify all of the subjects haplotypes to a specific allele, but the partially typed haplotypes can be determined using known population distribution alleles. Hence, the HLA DRβ1*0301/4 allele of HLA DR3 positive subjects is likely to be DRβ1*0301.

It is possible to predict which residues within peptide 49 - 63 bind to the HLA DRβ1*0301 molecule using known peptide sequences binding to this MHC molecule. The HLA DRβ1*0301 molecule has a binding motif characterised by the presence of a neutral hydrophobic anchor residue in positions 1 (L, I, M, V, F) and 9 (L, Y, F) and a hydrophilic residue at positions 4 (D, E) and 6 (R, N, K) (Rammensee *et al.*, 1995). Peptide 49 - 63 has an amino acid sequence LEIMIKFNRLNRLTT. Since the HLA DRβ1*0301 has a core binding motif of nine amino acids it is predicted that the epitope would begin within the first 6 residues of the peptide and at a hydrophobic amino acid. In addition, it is possible that during synthesis the peptide was not completed e.g. only a 13 mer, which further suggests that the epitope must begin early in the sequence 49 - 63. The epitope is therefore predicted as one of the following:

		Hydrophobicity/hydrophilicity at prominent positions:			
Residues	Sequence	P1	P4	P6	P9
49 - 57	**LEIMIKFNR**	phobic	phobic	philic	philic
51 - 59	**IMIKFNRLN**	phobic	philic	philic	philic
52 - 60	**MIKFNRLNR**	phobic	phobic	philic	philic
53 - 61	**IKFNRLNRL**	phobic	philic	phobic	phobic
55 - 63	**FNRLNRLTT**	phobic	phobic	philic	philic
1 - 9	preferred sequence	phobic	philic	philic	phobic

The binding of peptide to MHC molecules is largely independent of the peptide sequence and dependent on charge and hydrophobicity. A hydrophilic residue at position 4 in the peptide is highly favourable for binding to HLA DRβ1*0301. Hence, of the five motifs listed above it is predicted that peptides 51 - 59 or 53 - 61 would bind better to the MHC molecule. However, the majority of published peptide sequences shown to bind this MHC molecule have an aspartic acid (D) or glutamic acid (E) residue at position 4 (Rammensee *et al.*, 1995). Neither of the two predicted peptides have the preferred residues at this position.

In addition to using known peptide sequences to predict the binding motifs of peptides to MHC molecules a scoring system has been proposed for HLA DRβ1*0301 (Godkin *et al.*, 1998). The scoring system is based on 15mer peptides and stresses that peptide endings outside of the core binding motif are important in identifying T cell epitopes (Reece *et al.*, 1994). Peptides with a probability of being processed and presented (PPP) score nearer to two are more likely to bind the HLA DRβ1*0301 molecule. This scoring system was applied to the La protein (Godkin, 1999; personal communication). Within peptide 49 - 63 an epitope beginning at residue 49 gave the highest score (1.144). The scoring system predicts that position 1 of the core epitope does not occur until the following third or

fourth residue suggesting that the core epitope of the La peptide would begin at residue 52 or 53. Taken together with the prediction based on the review by Rammensee *et al.* (1995), it is predicted that the core epitope of peptide 49 - 63 would cover residues 53 - 61. This region covers the centre of the peptide and may explain why peptides shifted three amino acids to the left or right of peptide 49 - 63 failed to stimulate a proliferative response. Peptide 53 - 61 could be confirmed as the core epitope by using truncated peptides covering residues 49 - 63. Additionally, amino acid substitutions at each residue would define the critical residues required for binding to the MHC molecule and interacting with the TCR. The peptide stimulating maximum proliferation might be used to predict other peptides binding HLA DRβ1*0301 or to identify bacterial or viral epitopes which could initiate the immune response through molecular mimicry.

La peptides which bind better to HLA DRβ1*0301 than peptide 49 - 63 were predicted using the scoring system of Godkin *et al.* (1998). These include peptides 19 - 33, 127 - 141, 214 - 228 and 334 - 348. However, these peptides were not stimulatory *in vitro* (see Appendix C). Using the scoring system peptide 49 - 63 is within the top ten percent of the predicted binding peptides from the La protein. However, Godkin *et al.* (1998) reported known epitopes presented by HLA DR2, including MBP 84 - 102, fall in the top five percent of peptides taken from the protein (Godkin *et al.*, 1998). Nevertheless, it is difficult to identify the Hsp65 peptide 2 - 13 as a ligand for HLA DR3 using the PPP scoring system. It was suggested that this reflects the degeneracy of binding to class II molecules and the difficulty in predicting ligands using only the binding motif. Hence, if the scoring system is used for identifying stimulatory epitopes in a protein some *in vitro* stimulatory epitopes may be missed.

6.7.6 Naive and primed T cell responses

The CPM value and stimulation index do not necessarily reflect a naive and memory T cell responses since multiple epitopes from a single protein are likely to be involved in the response. Peptide responses are generally lower than proliferative responses to whole

protein. However, the percentage of replicating wells responding to the La peptides suggests that the proliferative response to the La peptide pools is mediated by naive T cells in healthy individuals and by primed T cells (albeit a low level response) in patients. Additionally, the response to peptide 49 - 63 occurs equally well in healthy individuals and patients and suggests that the response is mediated by the same T cell population. To clarify these findings proliferative responses to peptide pool 8 and peptide 49 - 63 were examined in purified CD45RA and CD45RO CD4 T cells (see section 6.5). CD45RA T cells and CD45RO T cells were presumed to reflect a naive and primed T cell population respectively.

Healthy individuals respond to rabies protein in the naive T cell population and tetanus toxoid (or PPD) predominantly in the primed T cell population but to a lesser degree in the naive T cell population. This is in agreement with Pilling *et al.* (1996) and Plebanski *et al.* (1992). The response to peptide pool 8 and peptide 49 - 63 occurred in the naive T cell population of the healthy individuals. This is in agreement with predictions obtained through the studies using PBMC's in the assay system. La specific T cells have therefore clearly not been deleted from the T cell repertoire during thymic tolerance. Similar findings have been suggested for other autoantigens stimulating proliferative responses in healthy individuals (Crow *et al.*, 1994; Kuwana *et al.*, 1995; Lohmann *et al.*, 1996; O'Keeffe *et al.*, 1999). This lack of tolerance may be due to the relatively low abundance of the La protein and its nuclear / cytoplasmic sequestration or to the peptides not being presented in the thymus. Alternatively, a state of anergy may been induced *in vivo* which was overcome *in vitro* with the addition of anti - CD28 monoclonal antibody. However, the proliferative response to peptides using PBMC's occurred in the presence and absence of the anti - CD28 monoclonal antibody.

It cannot be excluded that the CD45RA responding T cells are CD45RO revertant cells or that CD45RA cells represent resting T cells and CD45RO cells represent activated T cells (Bell and Sparshott, 1990). To confirm that autoreactive La specific T cells in the peripheral blood of healthy individuals represent a naive response the response in cord

blood would have to be examined. Autoreactive T cells in the periphery of healthy individuals possibly play an immune regulatory role which are normally kept under control. The mechanism associated with a loss of control of autoreactive cells is unknown.

In patients, proliferative responses were found to tetanus toxoid and PPD in CD45RO purified T cells. However, patients failed to respond to rabies protein in both the CD45RA and CD45RO T cells. However, patient HJ responded to peptide 49 - 63 in the primed T cell population and rabies protein in the naive T cell population with a SI greater than 2 but less than 3. As a consequence of the poor proliferative response to the antigens it is unclear if the response to the La peptides is mediated through the naive or primed T cell population. The predictions obtained through the studies using PBMC's in the assay system, that peptide pool 8 stimulated primed T cells and peptide 49 - 63 stimulated naive T cells in patients, were therefore not confirmed.

Proliferative responses to fractionated cells may be lost due to *in vitro* manipulation of the cells and irradiation of APC's prior to addition to the assay. Additionally, the role of B cells as APC's cannot be excluded since they were removed from assays using fractionated T cells. B cells present antigen to memory T cells but not to naive T cells (Croft *et al.*, 1992). This suggests that if B cells were responsible for presenting the antigen the response in patients to peptide 49 - 63 is mediated through memory cells. The role of the APC's could be investigated using non - irradiated macrophages or by adding back B cells to the assay. Alternatively, autologous EBV transfected B cells could be used as the APC. However, they could not be generated from patients responding to peptide 49 - 63. Additionally, IL7 has been used to grow out primed T cells specific for MBP in patients with multiple sclerosis. An increased T cell frequency in patients was reported compared to healthy individuals (Ms. B. Bielekova and Dr. R. Martin, 1999; personnel communication). Hence this method is worth investigating in SLE patients using the La peptides.

It is possible that a low level primed T cell response occurs in patients with SLE but goes

undetected using current proliferation assays (Byrne *et al.*, 1988). Altman *et al.* (1996) used a class I tetramer of HLA A2 complexed with gag or pol to reveal antigen specific T cell responses in the circulation of patients with HIV infection. A tetramer is a biotinylated MHC molecule folded around a peptide determinant and cross linked with streptavidin. This tetramer has higher affinity for the TCR compared with soluble antigen. Although this technique is more advanced with MHC class I bound antigens class II tetramers are beginning to be reported. It is therefore suggested that this technique is applied to the La peptides to increase the level of detection of antigen specific T cells.

Since the anti - La antibody response is class switched primed T cells specific for La were speculated to be present in patients with SLE. The present study does not confirm this hypothesis since the proliferative responses to the La peptides were poor. The lymphocytes may have been taken from the wrong site and / or at the wrong time; i.e. peripheral blood and not lymph nodes were used and the patients did not have active disease. Bach *et al.* (1997) report that the proliferative response to recombinant GAD declines over time in patients with IDDM. This suggests that T cell responses to La peptides may be found in SLE patients newly presenting to the clinic with the disease. However, this may also be too late to detect specific T cells involved in the pathogenesis of the disease.

6.7.7 Other approaches for measuring La specific T cells

The identification of a stimulatory peptide *in vitro* does not conclude that the peptide is important in the pathogenesis of the disease. Peptide 49 - 63 may be a cryptic epitope generated as a result of the stop / start positions of the synthetic peptides. To confirm that the peptide has a role in disease the peptide should be immunogenic when injected into mice. A good response *in vitro* but a poor response following immunisation suggests that the peptide represents a cryptic epitope (Reynolds *et al.*, 1996; Fairchild *et al.*, 1996). Alternatively, peptides eluted from the MHC molecule of EBV transfected B cells cultured with La protein could be examined for the peptide 49 - 63 (Chicz *et al.*, 1993). However, EBV transfected B cells are poor at processing and presenting whole antigen and the MHC

elution technique is laborious. Alternatively, the ability of La specific T cell lines to respond to the La peptide 49 - 63 may determine the value of the peptide response. Since generating T cell lines appeared to be the most practical of the three suggestions initial steps were carried out to evaluate if further work in this direction would be viable.

Human La was partially purified from the K562 cell line using the method of Stefano (1984). Proliferative responses to this partially purified La mirrored the response to peptide 49 - 63 in patients suggesting that the peptide is not a cryptic epitope. However, it cannot be excluded that the proliferative response was directed to contaminating proteins. Protein purified by the same method but from a K562 cell line which did not express La is suggested as a control for proliferative responses to the contaminating proteins. However, a cell line in which La has been genetically removed is not currently available. In addition, responses to peptide 49 - 63 were found in healthy individuals suggesting that La specific T cells are not deleted or irreversibly anergised during thymic and peripheral tolerance.

To continue these studies the La preparation used in these preliminary studies would require further purification and the proliferative responses re - examined. Since this would require extensive work attempts were made to stimulate cells with La bound to nitrocellulose membrane which was cut out from the contaminating proteins (Abou-Zeid *et al.*, 1987; Lamb *et al.*, 1988). However, tetanus toxoid bound to nitrocellulose membrane stimulated a poor response in patients compared to soluble tetanus toxoid so this avenue was not pursued further. Additionally, it was not possible to electroelute the protein from SDS PAGE protein gels since the contaminating bands were too close to La and could not be sufficiently separated using altered ratios of acrylamide : bisacrylamide (Buyon *et al.*, 1990).

6.7.8 Apoptosis reveals neo - epitopes of the La protein

It was presumed that human La and La peptides are processed *in vitro* in the same manner as *in vivo* and that they do not provoke new primary responses *in vitro*. However,

Markovic-Plese *et al.* (1995) showed that T cell lines specific for myelin proteolipid protein (PLP) recognise dominant peptide epitopes but that T cells specific for the dominant PLP peptides recognise additional epitopes. Additionally, Sjostrom *et al.* (1998) showed that deamidation of glutamine residues within gliadin are critical for the creation of active epitopes. These observations suggest that peptides undergo post translational modifications and has prompted a search for processes which induce novel fragmentation of autoantigens (Utz and Anderson, 1998).

It has been suggested that apoptosis may be responsible for the generation of autoantigens in SLE since autoantigens, including La and Ro, are found clustered in apoptotic blebs (Casciola-Rosen *et al.*, 1994a). Miranda *et al.* (1998) showed that Ro and La are translocated to the cell surface of the blebs providing further evidence that apoptosis is involved in SLE. More recently, Rutjes *et al.* (1999) report that La is dephosphorylated and specifically cleaved in the C terminus of the protein during apoptosis. Likewise, Utz *et al.* (1997) report that proteins selectively phosphorylated during apoptosis are targets for autoantibodies in SLE. Additionally, U1-70K protein (Casciola-Rosen *et al.*, 1994b) and type I keratins are specifically cleaved during apoptosis (Ku *et al.*, 1997). Hence, it is speculated that the apoptotic process and associated caspase enzymes cleave La at specific sites to reveal neo - epitopes. However, in the present study peptides predicted to result from caspase cleavage did not stimulate a proliferative response.

Apoptotic cells were present in the assay system used in the present study since approximately 10% of PBMC's die within 48 hours. However, the response to medium alone was not increased in patients when compared with healthy individuals. In contrast, Bell and Morrison (1991) showed that nucleosomes released spontaneously during short term cultures were stimulatory. Additionally, Voll *et al.* (1997a) report that apoptotic cells stimulate an increased production of anti-inflammatory cytokines by PBMC's *in vitro* compared to living cells. However, systemic exposure to apoptotic cells in mice did not induce anti - La antibodies although anti - dsDNA antibodies were detected (Mevorach *et al.*, 1998).

Since apoptosis is continually occurring in the thymus it is unclear why apoptotic material should be a major source of autoantigen in the development of SLE (Surch and Sprent, 1994). Herrmann *et al.* (1998) report a reduced clearance of apoptotic cells in SLE which may increase the quantity of La available to the immune system. Peptides eluted from MHC molecules are MHC related proteins or integral membrane proteins (Chicz *et al.*, 1993; Rudensky *et al.*, 1991). Few cytosolic peptides have been eluted. This suggests that La would not be presented in the thymus and, hence, the control of autoreactive T cells occurs in the periphery. Keratinocytes from patients with SLE show an increased susceptibility to UV light induced apoptosis (Casciola-Rosen and Rosen, 1997) and dendritic cells undergo apoptosis whilst delivering signals to T cells during antigen presentation (Kitajima *et al.*, 1996; Muller *et al.*, 1994). It would therefore be interesting to determine if UV light induced the formation of novel La epitopes in SLE. Additionally, since SLE occurs more frequently in the black population it would be interesting to examine the role of melanin in protecting the immune system in these patients.

6.7.9 Alternative candidate T cell autoantigens

As proliferative responses to La peptides and protein occur in healthy individuals and patients with SLE (see sections 6.4 and 6.6) it is suggested that La specific T cells are not specifically associated with the disease and are not deleted during thymic tolerance. Patients do not have serum anti - La antibodies in the absence of anti - Ro antibodies (Tomer *et al.*, 1993). This is possibly a result of La and Ro forming a complex with ribonucleoproteins (Mamula *et al.*, 1989). Additionally, Ro and La are found in the same apoptotic blebs in patients with SLE (Casciola-Rosen *et al.*, 1994a). Furthermore, T cells to Ro but not La have been detected in salivary glands taken from patients with SS (Namekawa *et al.*, 1995). These findings suggest that T cells which recognise Ro, or Ro complexed to La, are more important in the pathogenesis of SLE and this is where further research should be directed. For future studies, the Ro / La complex would have to be prepared by immunoprecipitation methods since Ro and La dissociate during column chromatography. Since nuclear antigens, including La, are 'sticky' it is also possible that

an unidentified protein may complex with La and that this complex is the T cell autoantigen in SLE.

As an alternative autoantigen it remains a possibility that a virus initiates the expansion of antigen specific T cells. Virus induced inflammation may result in the release of self antigen with cross reactive epitopes which perpetuates the disease. Zhao *et al.* (1998) report that HSV-1, associated with the destruction of corneal tissue in Herpes stromal keratitis, has sequence homology with keratin peptides. Additionally, the viral peptides can stimulate keratogenic specific T cell lines. Likewise, Coxsackie B4 virus has sequence homology with GAD and in mice infected with the virus autoreactive T cells to GAD are stimulated (Horwitz *et al.*, 1998).

HIV, CMV, HTLV and EBV are reported to be associated with SLE (Herrmann *et al.*, 1996; Nakamura *et al.*, 1998; Incaprera *et al.*, 1998). La protein has also been reported to associate with small virus RNA from EBV and adenovirus (Lerner *et al.*, 1981) and vesicular stomatitis virus (Kurilla and Keene, 1983). Furthermore, La is redistributed within the cell following infection with poliovirus, adenovirus or cytomegalovirus (Meerovitch *et al.*, 1993; Baboonian *et al.*, 1989). The La peptide 81 - 101, which is recognised by serum containing anti - La antibodies, has homology to retroviral gag peptides (Kohsaka *et al.*, 1990). Hence, a search of the protein databases was carried out on the stimulatory peptide identified in the present study, La 49 - 63 (Dr. S.P. Young, 1999; personnel communication) and a stealth virus was identified with 63.6% homology to La 49 - 63.

La 49 - 63	L	E	I	M	I	K	F	N	R	L	N	R	L	T	T
					|	|	|			|	|			
Stealth virus 115 - 129	A	A	N	A	I	K	F	D	D	L	N	K	M	T	T

The predicted core peptide 53 - 61 is underlined in the diagram above which shows the

homology between La 49 - 63 and stealth virus 115 - 129. There is good correlation at the beginning of the core peptide (residues 53 - 55). Additionally, the stealth virus has the preferred aspartic acid (D) residue at position 4 of the MHC binding motif. The stealth virus is reported to be a human pathogen. Martin (1996) report a stealth virus similar to CMV in individuals with chronic fatigue syndrome. Hence, this virus may be involved in the initiation of SLE.

This study was the first step towards determining if La is implicated in the T cell responses in SLE. What has evolved is a solid basis on which to continue investigating cell mediated response to nuclear antigens. Many of the findings in this study also suggest new avenues of research which may help to unravel the mechanisms associated with SLE.

Chapter 7

Conclusion and Future Work

In general, patients with SLE and / or APS showed a poor proliferative response to the standard antigens, PHA, tetanus toxoid, PPD and rabies protein, compared to healthy individuals. Even with prior exposure, i.e. vaccination, patients were unable to mount a proliferative response *in vitro* to the same magnitude as healthy individuals. However, the ability to respond to an antigen *in vitro* varies within the groups of subjects. In patients, the ability to respond to a standard antigen was not associated with HLA haplotype, serum autoantibodies, disease duration or medication.

Despite the reduced proliferative responses to standard antigens, a proportion of patients with SLE and / or APS showed a proliferative response to β_2GP1 *in vitro*. Healthy individuals did not respond to β_2GP1 *in vitro* suggesting that β_2GP1 plays a role in disease pathogenesis. However, in patients, there was no clear association between the proliferative response to β_2GP1 and the presence of serum antibodies to β_2GP1, antibodies to phospholipids or disease duration. Furthermore, there was no clear association between the ability to respond to β_2GP1 and HLA haplotype. However, only the HLA DRβ1 and HLA DQβ1 alleles were typed so the response may be restricted by other MHC molecules.

The mean proliferative response, in terms of the CPM value, comparing β_2GP1 and rabies protein as the antigens, suggests that the response to β_2GP1 is mediated by naive T cells in responding patients. However, since only a few patients were capable of responding to rabies protein, compared with healthy individuals, the proliferative response to β_2GP1 may be more significant than the CPM values suggest. It is speculated that β_2GP1 specific T cells in patients have not fully differentiated into primed T cells and, hence, can only achieve a low level response. For future work it is therefore necessary to define what a positive response by patients is (in terms of CPM value or relationship to other antigens) compared with healthy individuals. This may also give some clues as to the mechanisms

associated with the disease.

Healthy individuals and patients with SLE and / or APS showed a proliferative response to La peptides and partially purified human La. A single peptide (peptide 49 - 63) was found to be the dominant stimulatory peptide and was restricted by the HLA DR molecule. However, HLA DQ appears to play a role in the proliferative response to other La peptides. There was no obvious association between the proliferative responses to the La peptides and the presence of serum anti - La antibodies. Future work on the La protein is largely dependent on determining if the stimulatory peptides are immunogenic and derived *in vivo* through the processing of the La protein. The present study has laid the foundations for continuing this work by examining the feasibility of preparing T cell clones specific to La.

Comparing the data of the proliferative responses to the La peptide pools with the responses to rabies protein suggests that the response to the La peptide pools is mediated by naive T cells in healthy individuals and by primed T cells in patients. However, the response to peptide 49 - 63 appears to be mediated by naive T cells in both healthy individuals and patients. In an attempt to confirm these suggestions the response to the peptides was examined in purified populations of CD45RA (naive) and CD45RO (primed) T cells. The data showed conclusively that in healthy individuals the response to the stimulatory peptides, peptide 49 - 63 and the peptide pool 8, was mediated by naive T cells. However, the response to standard antigens in fractionated T cells taken from patients was very poor. Hence, a clear answer regarding the population of T cells mediating the proliferative responses to the La peptide pools in patients could not be determined.

Further evidence is required to determine the nature of the β_2GP1 and La specific T cells and to determine if any differences occur between healthy individuals and patients. As the first step in answering this important question it is suggested that the experiments carried out in the present study using CD45RA and CD45RO purified T cells be repeated but

using β_2GP1 and La as the antigen. However, it is suggested that irradiated EBV transformed B cells, allogeneic adherent cells or PBMC's are used as the antigen presenting cells. EBV transformed B cells are good at presenting peptides but poor at presenting proteins to T cells. Hence, using these cells as the APC's may not prove fruitful. Allogeneic preparations overcome any problems associated with defects in APCs of patients with SLE. However, cells must be tissue type matched to minimise the effects of a MLR. PBMC's offer the advantage of overcoming problems associated with removing important cell populations from the experiment. It may not be necessary to irradiate the adherent APC's before adding to the cultures. However, any proliferation of the APCs will contaminant the purified T cell populations making it difficult to interpret the data. Alternatively, limiting dilution analysis (LDA) would indicate any differences in T cell frequency between healthy individuals and patients. However, LDA has been shown to underestimate the T cell frequency and may not prove to be valuable.

T cell responses to β_2GP1 and La in patients appear to be involved in the pathogenesis of SLE and / or APS but the mechanism remains unclear. The classic immunological dogma suggests that T cells activate B cells of the same specificity to produce antibody. However, there is no clear association between the proliferative response to La or β_2GP1 and the presence of serum antibodies. Hence, it is speculated that the T cells and B cells involved in the disease have different protein specificities. It is hypothesised that T cells in patients with SLE and / or APS have been activated but have not correctly switched from the naive to primed phenotype. This is supported by data showing that T cells from patients with SLE are refractory to further stimulation *in vitro*, although increased cytokine levels have been reported. In addition, the autoantibodies associated with the disease are directed against specific antigens suggesting that the autoimmune response is regulated or that the dysfunction is specific to the antigen(s). It is therefore important that the collaboration between T cells and B cells and the relevance to the production of antibodies is examined in future work.

The majority of peptides bound in the class II MHC binding groove are derived from the

MHC molecule or from extracellular proteins. The location of β_2GP1 and La suggests that they are processed and presented in an abnormal manner during an autoimmune response. One possibility is the production of new epitopes through apoptosis. However, apoptosis occurs in all individuals and it is unclear why epitopes produced in this manner, if they are produced through apoptosis, should be involved in autoimmunity. The process of apoptosis may reveal antigens which are normally sequestered in the nucleus or in an inappropriate form for being taken up by the APC's. Alternatively, the failure to clear apoptotic bodies in SLE patients may simply increase the abundance of antigen available to the immune system.

Alternatively, it is speculated that the antigen may be bound to another protein. This could potentially alter the peptide / protein conformation and reveal cryptic epitopes. For the La protein the obvious candidate protein is Ro since Ro and La form a complex *in vivo*. For β_2GP1 it is postulated that protein S, protein C or other cofactors, bind the protein and phospholipids and that this complex drives the immune response. In future work to continue these studies it is therefore proposed that the proliferative response to these protein complexes is examined. However, it cannot be excluded that non - self antigens are involved in the activation of self reactive T cells.

Antigens are presented to T cells in the context of the MHC molecule on the surface of DC's, macrophages and B cells. These APC's were not specifically examined during the present study. However, the data obtained in the present study suggests that B cells may play an important role in antigen presentation. One possibility is that B cells support the role of macrophages, since macrophages are reported to be defective in patients with SLE. B cells from patients with SLE may present antigen to both naive and primed T cells. Alternatively, antigen may be taken up by B cells via normal phagocytosis but processed differently which leads to the generation of altered epitopes. In addition, complement may enhance the uptake of antigen bound antibody complexes which augments the response to the antigen. However, in SLE there is a deficiency of early complement factors. The role of B cells and complement were not examined in the present study. However, since they

may play a role in the immune response to β_2GP1 and La future studies are suggested in this direction.

β_2GP1 and La specific T cells may arise as a consequence of the disease process and generalised cell dysfunction. However, this does not account for their presence in healthy individuals. The presence of β_2GP1 and La specific T cells with the array of other cell defects may however lead to the perpetuation of the disease. The general dysregulation may reflect the different symptoms in patients fulfilling different disease criteria, and that SLE is a syndrome and not a single disease. It will therefore be important to clearly define the patient groups based on symptoms and serology in future studies.

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Appendix A1

Details of healthy individuals (* refers to male subjects) enrolled onto the studies listing:

- date of birth
- BCG vaccination
- tetanus toxoid vaccination (+ = vaccinated more than 10 years ago, ++ = vaccinated within the last 10 years)
- serum anti-tetanus toxoid antibody titre (μg/ml)
- serum IgG titre (mg/ml)
- T cell proliferative responses (thymidine incorporation, CPM) to medium, tetanus toxoid, PPD, rabies protein and PHA in individual patients
- HLA type

	Date of	Vaccination		TT Ab	IgG
Name	birth	BCG	TT	ug/ml	mg/ml
NA	21.9.64	+	++	128.4	18.2
GB	20.7.44	+	+	56.1	11.5
GBA	3.2.56	+	++	75.3	15.3
PB*	27.6.69	+	++	141.6	8.9
PBA	10.1.46	+	+	106.4	8.4
BC	2.6.65	+	++	56.3	12.1
CC*	2.6.72	+	++	140.6	13.1
DC*	11.2.65	+	++	49.1	13.2
MD	25.10.71	+	++	139.5	13.7
MDO*	3.10.70	+	++	133	18.7
JF*	4.2.67	+	+	118.1	19.8
AG	21.10.51	+	++	5	13.4
CG	1.11.56	+	++	133.8	14.6
GG	18.7.62	+	++	98.6	11.2
JG	22.9.63	+	+	49.4	13.7
GH	2.10.46	+	+	139.5	8.7
SH	17.12.68	+	++	106.7	21.9
LI	31.5.63	+	++	65.3	19
DJ	3.3.67	+	++	120.4	15.9
JK	8.7.53	+	++	20.8	8.7
PK	21.6.49	+	++	20.7	16.2
CL	3.4.56	+	+	51.4	7.6
JL	29.1.72	+	++	117.3	10
ML	26.10.40	+	++	75.5	10.8
BM	1.3.56	+	++	42.5	19.9
JM*	10.7.65	+	+	128.8	11.2
DP*	30.6.64	+	++	133.8	12.6
CR	24.4.71	+	++	116	20.4
DS	3.3.64	+	++	107.4	8.7
HS	25.11.69	+	++	141.5	22.7
JS	23.2.48	+	++	9.7	19.7
PS	3.10.50	+	++	70.2	11.3
HU	17.4.47	+	++	67.9	17.7
BW	18.2.48	+	++	132.4	19.6
PW*	15.1.61	+	++	71.3	14.6

		T cell proliferation (CPM)						
Name	Med	TT	PPD	Rabies	ß2GP1	PHA	HLA DR	HLA DQ
NA	238	88164	2890	388	289	114326	0301, 1501	0201/2, 0602
GB	603	7369	55415	684	ND	31016	0101/5, 1302	0501-4, 0604-9
GBA	173	4576	83065	495	ND	150384	0101/5, 1101	0501-4, 0301
PB*	217	42937	23026	1294	ND	11185	0301/4	0201/2
PBA	157	5218	75627	765	336	19618	1302, 1501	0501-4, 0603/14
BC	939	7507	32087	976	ND	112185	0101/2/4	0501-4
CC*	608	24279	39557	614	ND	83561	16, 0701/3	0201/2, 05
DC*	492	10731	56925	598	ND	36130	1, 14	*05
MD	544	61978	41661	2551	704	94576	1501, 0701	02, 0602
MDO*	910	97042	9604	1249	ND	30351	04, 1401/7	05, 0302
JF*	694	41590	91246	3851	ND	70108	0301/4	0201/2
AG	520	17249	64332	3327	880	67667	1501, 0401-22	0602, 0301/4
CG	263	6230	18670	1753	572	21496	0102/4, 0701/3/4	0501-4, 0303
GG	431	929	29995	502	ND	12638	0101/5, 1101/4/6	0501-4, 0301
JG	249	1138	8329	1063	420	23546	0301, 1501	0201/2, 0602
GH	159	701	970	564	168	25461	1102/3, 1301	0603/4, 0301/4
SH	786	17238	119102	789	793	113329	1302, 1201-3	0604-9, 0301/4
LI	174	95164	1616	486	196	59045	nd	nd
DJ	874	21386	56327	1153	ND	40082	0101/5	0501-4
JK	240	5963	7275	731	ND	64352	0101/5, 08	0401/2, 0501-4
PK	441	1414	5490	722	ND	52810	1501, 0301	0602, 0201/2
CL	599	5900	54671	5280	ND	75748	0101/5, 1302	*0604
JL	645	2421	2512	6503	678	679	0101/5, 1201-5	0301/4, 0501-4
ML	514	37832	14651	1745	2018	109472	0102, 0701	0201/2, 0501-4
BM	515	1814	8403	1637	559	28437	0301, 1401/7	0201/2, 0501-4
JM*	615	17131	11053	1504	ND	53302	0301/4, 1301/16	0201/2, 0603
DP*	471	4784	36575	1103	ND	65773	0301/4, 1301/16	0201/2, 0603/14
CR	727	83241	97172	1736	838	110645	*0103	*05
DS	141	161	98761	1282	357	46329	0301, 1301	0201/2, 0603/14
HS	661	59397	39229	1345	710	84961	04, 0701/3/4	0201/2, 0302
JS	993	2413	31131	1354	1617	31540	0801-11, 1301/16	04, 0603
PS	559	23368	5657	918	ND	62073	0301/4	0201/2
HU	444	70355	49951	3279	ND	77611	0301/4, 04	0201/2, 0302
BW	822	25712	11756	2376	ND	94627	0301/4, 1401/7	0201/2, 0301/4
PW*	385	13247	8702	315	524	64907	11, 1302	0604, 0301

Appendix A2

Details of patients (* refers to male patients) enrolled onto the studies listing:

- date of birth
- diagnosis and date
- serum autoantibodies (R = anti-Ro autoantibody, L = anti-La autoantibody, A = anti-cardiolipin antibody, D = anti-DNA antibody, + = renal disease)
- medication during the last year (H = hydroxychloroquine, P = prednisolone, A = azathiopine, M = methotrexane, C = cyclosporin)
- BCG vaccination
- tetanus toxoid vaccination (+ = vaccinated more than 10 years ago, ++ = vaccinated within the last 10 years)
- serum anti-tetanus toxoid antibody titre (μg/ml)
- serum IgG titre (mg/ml)
- T cell proliferative responses (thymidine incorporation, CPM) to medium, tetanus toxoid, PPD, rabies protein and PHA in individual patients
- HLA type.

§ denotes patients not included in the studies comparing the proliferative response to previous exposure since their vaccination status was not determined (see section 4.3).

Patient	Date of	Diagnosis	Auto Ab	Medication	Vaccination		TT Ab	IgG
	birth				BCG	TT	ug/ml	mg/ml
KA	21.6.76	SLE/APS 1995	A +	P, A	+	+	136.5	36
JA	28.6.42	SLE 1964		H, P, A	+	++	14	15.9
SA	1.8.45	SLE 1984			+	+	0	19.3
PBA	6.8.46	SLE 1963	+	P, M	+	++	11	28
MB	17.10.41	SLE 1994	R, L	H, P, A	+	++	10.9	42.3
JB	13.6.56	SLE 1976			+	+	72.6	13.9
EB	7.5.33	SLE 1990			-	-	27.3	16.8
MBO*	15.1.46	SLE1997			+	+	27.2	18.1
KB	27.8.71	SLE 1994	A	P, A	+	-	11.9	31.7
KBR	15.6.69	SLE 1984	+/-	H, P, A	+	+	0	31.3
AB	20.5.49	SLE / RA 1986	R, L, A, D	P, C	+	+	14.8	18.4
PBR §	ND	SS			ND	ND	ND	ND
CB	25.12.37	SLE 1947	D		-	+	0.75	41.3
NC	29.9.35	SLE 1980	+/-	P, M	-	++	16	17.9
GC	2.6.38	SLE 1986	R, L	P, A	-	++	16.8	25.7
HC	20.8.60	SLE 1985	A, D +	H, P	+	++	136.5	24.2
JC	24.4.45	SLE 1987	R, L, A, D +	P	+	++	15.6	15.9
HCO	28.6.73	SLE 1989	R, L +	H, P, A	+	++	9	25.3
AD	1.3.75	SLE 1994	D +	P, C	+	++	70.2	22.6
KD	21.5.64	SLE 1981	D		+	++	52.2	15.9
DD*	28.11.34	SLE 1995	D +/-	H, P	+	++	62.4	13.2
DDY	25.10.27	SLE 1993		P	-	-	0	28.1
GE*	3.9.65	SLE 1994		H	+	+	55.5	26.1
SF	16.11.67	SLE 1991	+	P, C	+	++	118.5	18.5
BF	27.6.44	SLE 1988	R, L	P	+	+	34.6	11
CF	13.3.64	SLE 1987		P, C	+	++	94.9	19.7
AF	17.3.48	SLE 1983	R	H	+	++	141	21.7
CFR	20.7.70	SLE 1990	A, D	P, A	+	+	117.9	3.4
JF	21.7.43	SLE 1965	A	P	-	-	10.6	11.9
BG	19.7.59	SLE 1985	R, D +	H, P	+	+	128.2	17.2
BGR	10.7.47	SLE 1976	+	P	+	++	64.1	14
CG	16.4.70	SLE 1990	R	P	+	++	112.5	14.5
TH	31.8.42	SLE 1991	D	H, P	+	+	48.9	21.2
NH	16.5.69	SLE 1994	A +/-	P	+	++	112.7	26.3
MH	10.11.35	SLE 1975	R, L, A, D	P, A	-	+	14.9	28.7
RHO	13.3.73	SLE 1989	A +	H, P, A	+	++	117.5	13.8
SH	6.9.46	SLE 1989	R, A	P, M	-	++	103	13.9
IH	22.8.24	SLE 1996	R, L	P	-	-	0.9	19
KH	20.1.69	SLE 1987	D	P	-	++	44.8	24.8
LH	27.1.69	SLE 1983	D	P	+	++	137.4	25.5
RHU	17.12.69	I°APS 1996			+	-	27.2	18.7
LJ	19.2.64	SLE 1981	A	P, A	+	++	122.5	19.6
AJ	18.7.68	SLE 1993	A +	P, A	+	+	64.5	13.4
HJ	18.12.73	SLE 1993	R, L, D	P, A	+	++	135.5	18.3
PK	14.7.48	SLE 1981	R, L	H, P	-	+	31.9	10/15.5
RK	27.11.53	SLE '77/APS '90	R, A, D	P, A	+	+	14.6	28.9
HK	2.7.29	pSS 1997			-	-	4.4	23.1
HL	29.12.43	SLE 1985	+	P, A	+	++	60.5	10.2
KL	1.1.60	SLE 1984	A		+	+	74	18.3
ML	10.4.39	SLE 1980/ SS	A	H	-	++	809	10
PL	19.3.46	SLE 1981	+	P	+	++	3	11.4

Patient	Date of birth	Diagnosis	Auto Ab	Medication	Vaccination		TT Ab	IgG
					BCG	TT	ug/ml	mg/ml
AL	22.12.37	SLE 1968	R, L, D		+	++	117	13.8
JMA	12.3.73	SLE/SS 1989	R, L +/-	P, A	+	++	101.2	15.2
HM	1.12.49	SLE/SS 1984	R, L, A, D	H, P	+	++	6.8	17.8
SM	1.8.67	SLE 1977	D +	H, P, A	-	-	64.7	14.5
PM	7.12.67	SLE 1988	A, D +	H, P, A	+	+	35.5	17.2
HMO	28.2.66	SLE 1987	+	P, A	-	++	91.5	17.4
CM	1.7.47	SLE 1984	D		+	-	30.9	18.4
BM	14.6.48	APS/SLE			+	+	49.3	21.6
HN*	1.2.49	SLE 1990	D +	P, C	+	++	117.9	14.2
MN §	ND	I°APS			ND	ND	ND	ND
JO	16.6.42	SLE 1982		H, P	?	+	83	15.6
JOR	23.2.43	SLE '63/APS '76	A, D +/-	H, P	+	+	131	19.2
CO	13.4.47	I°APS 1990	A		+	+	12	19.4
SO*	7.3.53	SLE 1994	+/-	H, P, C	+	+	9.3	10.1
AP	12.1.59	SLE 1986			+	-	83.5	17
JP	22.8.47	SLE 1980	R, L	H, P	+	+	9.4	19.7
BP	9.2.58	SLE 1986	D	P	+	+	55.3	15.5
GP	1.5.56	SLE 1976	R, A +/-	H, P, M, C	+	-	1	23.6
JPE	3.7.47	SLE 1971	+	H, M	+	++	46.5	15.1
JPH	10.3.58	SLE 1995	R, D	H, P, M	+	-	11.6	13.9
JPI	22.6.62	I°APS 1997	A		+	+	45	13.6
GPL	14.5.23	SLE 1959	R +/-	P	-	++	69.4	14.8
DP	1.8.49	SLE 1978	R, L +	P	+	++	95.8	10
APO	26.6.44	SLE 1983	R, L, D	H, P	+	++	50.6	11.7
YR	21.6.68	SLE 1979		H, P	+	+	59.4	16.3
MR*	12.11.49	I°APS 1990	A		+	++	54.9	21.7
MDR	22.1.33	SLE 1975	D +/-		-	++	118	17.1
LR	16.3.63	SLE 1968	R, D	P	+	+	48.4	16.2
RR	28.9.68	SLE 1992	D +	H, P, A	+	+	16.1	18.8
AS	19.1.65	SLE 1993	D +	P, A	+	+	128.3	17.8
SS §	ND	I°APS			ND	ND	ND	ND
SuS	14.7.49	SLE 1975	D, A	P, M	+	++	116.5	19.6
MS	7.4.47	SLE 1987	D	H, P	+	+	52.9	13.2
MSH	25.9.62	SLE 1985	D		+	+	58.1	14.6
ACS	25.2.45	I°APS 1994	A		+	++	17.4	19.5
RS	16.8.68	SLE 1986	R, A +/-	H, P	+	+	111.4	14.7
KS	10.12.57	SLE 1995		P	+	++	31.5	18.5
AT	17.9.51	SLE 1983	R, L, A	A	-	++	140.5	18.9
JT	29.11.44	SLE 1985	D		+	++	109.9	31.9
ATO	8.1.43	SLE 1963		H, P	+	++	70.9	26.4
MT	21.11.63	SLE 1984	D +	P, A	-	++	115.7	11.4
ST	13.8.66	SLE 1983		H, P	+	-	129.5	10
LV	4.9.47	SLE 1977	R, L, D +	H, P	+	++	128.7	14.4
DW	4.12.65	SLE/SS 1992	R, L +/-	H, P, A	+	++	54.6	18.7
MW	4.4.55	SLE 1983	D +	A	+	++	131	15
LW	2.12.50	SLE 1982			+	++	46.9	11.5
NW §	ND	I°APS			ND	ND	ND	ND
HW	15.3.73	SLE 1994	D		+	++	68.6	42.4
TW §	ND	I°APS			ND	ND	ND	ND
AW	28.12.35	SLE 1980			-	++	8.3	15.4
JW	11.7.63	SLE 1995	A +	H, P, A	+	+	64.8	17.6
LWR	20.10.68	SLE 1990		P	+	+	110.7	17.3
LY	30.1.60	SLE 1989	A, D	P	-	+	81	17.6

Patient		T cell proliferation (CPM)						
	Medium	TT	PPD	Rabies	ß2GP1	PHA	HLA DR	HLA DQ
KA	481	9658	32756	529	1023	61875	0101, 0401-22	05, 0301/4
JA	181	317	1378	236	211	11185	1301, 1303	0603, 0301/4
SA	356	535	36553	451	460	64445	1601-6, *0301	0201/2, 0501-4
PBA	632	2934	6857	1313	ND	10028	0401, 0404	*0302
MB	610	31544	15537	631	2763	47863	1501, 0301	0201/2, 0602
JB	802	16395	22994	838	1139	29286	1501-5, 0301	0601/2, 02
EB	645	1042	14082	3281	ND	37456	0401, 1501	0602, 0302
MBO*	391	731	6478	2659	4872	43479	0701, 1302	0201/2, 0604-9
KB	1739	14411	23948	2837	2246	48602	*0301	*0201/2
KBR	256	316	200	311	ND	30307	*0404	*0302
AB	181	229	602	221	426	134350	1501, 0301	0201/2, 0602
PBR §	345	1624	950	487	ND	54308	0301, 0801-11	0201/2, 0401/2
CB	215	284	1376	237	695	15642	1501, 0401	0602, 0301/4
NC	702	4021	14921	605	ND	8254	0101/2/4, 1301/2/8	05, 0604-9
GC	396	32204	14354	274	ND	27134	1501, 0301	0201/2, 0602
HC	942	44196	56786	1327	ND	9542	*0304	*02
JC	396	413	30717	474	ND	11516	0301, 0701	0201/2
HCO	778	1666	1817	2208	793	14763	0101, 0301	0201/2, 0501-4
AD	410	616	7875	444	574	69819	1501-5, 0901	*06, 09
KD	163	7443	3996	195	619	13805	0101, 0401-22	0501-4, 0301/4
DD*	940	1442	25811	2211	ND	20795	*0701	*02
DDY	277	310	177	254	ND	42167	0401-22, 1501-5	02, 05
GE*	912	13051	15835	1066	ND	19916	1301/2/8, 0304	02, 0603
SF	201	67010	40051	2924	ND	33162	0701, 1501	02, 0603
BF	202	734	4057	699	ND	44232	0301, 1301	0201/2, 0603
CF	191	2278	597	297	326	9617	1501	602
AF	742	8124	2466	1377	803	14312	1501-5, 0801-11	04, 0602
CFR	974	27275	16033	920	ND	23534	1304/6, 0304	02, 0604-9
JF	632	1498	3786	689	841	11638	0401, 1302	0604-9, 0301/4
BG	117	126	106	464	ND	14493	0404, 0304	02, 0302
BGR	830	17448	2785	840	904	15339	0101, 0301	0201/2, 0501-4
CG	997	45282	32589	1781	ND	35270	1501, 0301/4	0201/2, 0602
TH	309	698	1743	347	ND	44278	0101/2/4, 0401-22	05, 0301/4
NH	374	1076	4985	855	591	56039	0304, 1101/4/6	02, 0301
MH	114	172	2667	130	138	24174	0701, 1401/7	0201/2, 0501-4
RHO	574	7958	3986	583	638	25311	*0901, 0304	02, 0303
SH	870	10363	6562	1603	1100	72420	0401/4	0301/4, 0302
IH	115	172	23908	232	ND	8099	0401-22	0301/4, 0302
KH	202	910	246	266	239	94272	0701, 1501	0303, 0602
LH	803	27648	2168	521	ND	67041	0401, 1501	0601/2, 0301/4
RHU	305	638	9940	506	610	29245	0701, 1301	0201/2, 0603
LJ	986	7687	13795	1004	2444	42709	0101/2/4, 0401	05, 0302
AJ	259	6842	8973	543	379	19737	0404, 0301	0201/2, 0302
HJ	881	17527	3168	2697	9449	38611	1501, 0301	0201/2, 0602
PK	433	1306	8351	559	1371	12584	1301, 0301	0201/2, 0603
RK	139	296	629	186	267	32173	0401, 0301	0201/2, 0301/4
HK	178	185	1631	209	ND	53551	0301, 08	0201/2, 0301/4
HL	276	831	11931	234	ND	2010	1401/7/16, 0301	02, 05
KL	472	948	2014	481	506	16379	0402, 13	0301/4, 0302
ML	846	25973	28380	2945	ND	72435	13, 1201-3	0604-9, 0301/4
PL	392	649	12269	574	ND	21707	0701, 0304	*02

Patient		T cell proliferation (CPM)						
	Medium	TT	PPD	Rabies	ß2GP1	PHA	HLA DR	HLA DQ
AL	318	31478	4075	3148	ND	33678	*0301	0201/2
JMA	497	531	976	731	542	21431	0801-11, 0301	0201/2, 0401/2
HM	222	318	637	304	384	12437	*0301	*02, 02
SM	805	6746	5724	833	ND	79284	0701, 1307/21	02, 0301/4
PM	910	8535	22489	2533	2568	67315	0401, 0304	02, 0302
HMO	140	223	1429	730	875	4950	1501	*0602
CM	289	13618	58621	875	ND	41625	0401, 0408	0301/4, 0302
BM	186	258	198	201	ND	16551	15, 0301	0201/2, 0602/10
HN*	377	49025	26373	399	ND	53415	1303, 1316	0603, 0301/4
MN §	529	7070	35452	1602	1631	91963	0301, 1302	0604-9, 0201/2
JO	582	28784	1989	832	ND	70963	0701, 0801-11	04, 03032
JOR	945	3881	9127	4283	2493	63752	1501, 1401/7	05, 0601/2
CO	965	13726	84784	2246	4697	43109	0301, 0701	*02
SO*	957	28313	47249	1959	ND	49168	*0304	*02
AP	513	6904	1439	671	535	32741	0401, 1301/16	0603, 0301/4
JP	278	5569	2971	406	ND	36623	0301, 13	0201/2, 0603
BP	213	469	1173	374	ND	47402	0801-11, 1501	04, 0601/2
GP	197	286	204	219	235	26397	1001, 1302	0501-4, 0604-9
JPE	334	1848	33508	2525	ND	78088	0101/2/4	*05
JPH	171	195	1581	216	ND	3856	*0301/4	*0201/2
JPI	563	987	5034	715	1256	9996	1501, 0401-22	0602, 0302
GPL	570	2180	731	595	ND	66852	0103, 0304	05, 02
DP	601	643	43999	869	717	17630	11, 0301	0201/2, 0301/4
APO	211	3064	9660	675	732	66071	0301, 1302	0201/2, 0604-9
YR	406	423	5147	636	ND	20107	0401, 1302	0604-9, 0301/4
MR*	804	17452	32551	1116	1286	88248	0301, 0401-22	0301/4, 0201/2
MDR	1515	21154	12597	7148	3734	63709	0101, 0301	0201/2, 0501-4
LR	179	188	668	384	301	11930	0301, 0801-11	0201/2, 0401/2
RR	982	2870	22525	1028	ND	13912	*0304	*02
AS	192	124	142	263	ND	4174	1501, 0304	02, 0601/2
SS §	473	2597	3596	602	484	5281	0401-22	*0302
SuS	106	1221	240	344	233	7233	*0301	0201/2
MS	448	2018	7326	645	ND	8119	0701, 11	02, 0301/4
MSH	631	7702	25011	437	ND	64828	1501, 0304	02, 0602
ACS	639	40892	22224	844	2712	59142	1301, 0401	0603, 0301/4
RS	352	2513	503	353	968	26886	0103, 0901	0201/2, 0501-4
KS	212	321	1361	191	ND	37429	0801-11, 1302	04, 0604-9
AT	396	22003	10140	543	421	77949	0301, 0701	*0201/2
JT	336	16105	24223	289	ND	26069	0101/2/4, 1302	05, 0604-9
ATO	609	15462	7255	621	ND	4765	0701, 1501	02, 0602
MT	353	1889	390	261	ND	8293	1501-5	0601-3
ST	196	741	1116	574	275	12748	0103, 0301	0201/2, 0501-4
LV	475	33077	27010	1948	3482	78325	0301, 11	0201/2, 0301/4
DW	196	4960	525	198	284	42318	1302, 0301	0201/2, 0604-9
MW	252	56513	933	762	ND	82953	1501, 13	02, 0601/2
LW	464	61674	27027	429	ND	33859	1501, 0304	02, 0601/2
NW §	681	8656	2965	702	770	16983	0301, 0701	0201/2
HW	533	1198	1216	126	ND	19544	1501-5, 0701	0601/2, 3032
TW §	362	26407	1182	1898	2080	23942	1501, 1301	0601, 0603
AW	646	42397	28540	3130	750	67080	0701, 0301	*0201/2
JW	652	39677	25451	1833	2176	38261	*0304	*02
LWR	232	8521	9716	572	ND	26829	0304, 0401-22	02, 0301/4
LY	663	1794	2232	1837	3754	8599	0401, 0701	02, 0601/2

Appendix B1

Alphabetical list of the naturally occurring amino acids

Amino Acid	**Three letter Abbrev.**	**One letter symbol**	**Physical status**	
Alanine	Ala	A	neutral	hydrophobic
Arginine	Arg	R	basic	hydrophilic
Asparagine	Asn	N	neutral	hydrophilic
Aspartic acid	Asp	D	acidic	hydrophilic
Cysteine	Cys	C	neutral	hydrophobic
Glutamine	Gln	Q	neutral	hydrophilic
Glutamic Acid	Glu	E	acidic	hydrophilic
Glycine	Gly	G	neutral	hydrophobic
Histidine	His	H	basic	hydrophilic
Isoleucine	Ile	I	neutral	hydrophobic
Leucine	Leu	L	neutral	hydrophobic
Lysine	Lys	K	basic	hydrophilic
Methionine	Met	M	neutral	hydrophobic
Phenylalanine	Phe	F	neutral	hydrophobic
Proline	Pro	P	neutral	hydrophobic
Serine	Ser	S	neutral	hydrophilic
Threonine	Thr	T	neutral	hydrophilic
Tryptophan	Trp	W	neutral	hydrophobic
Tyrosine	Tyr	Y	neutral	hydrophobic
Valine	Val	V	neutral	hydrophobic

Appendix B2

Amino acid sequence of humam La protein

MAENGDNEKM	AALEAKICHQ	IEYYFGDFNL	PRDKFLKEQI 40
KLDEGWVPLE	IMIKFNRLNR	LTTDFNVIVE	ALSKSKAELM 80
EISEDKTKIR	RSPSKPLPEV	TDEYKNDVKN	RSVYIKGFPT 120
DATLDDIKEW	LEDKGQVLNI	QMRRTLHKAF	KGSIFVVFDS 160
IESAKKFVET	PGQKYKETDL	LILFKDDYFA	KKNEERKQNK 200
VEAKLRAKQE	QEAKQKLEED	AEMKSLEEKI	GCLIKFSGDL 240
DDQTCREDLH	ILFSNHGEIK	WIDFVRGAKE	GIILFKEKAK 280
EALGKAKDAN	NGNLQLRNKE	VTWEVLEGEV	EKEALKKIIE 320
DQQESLNKWK	SKGRRFKGKG	KGNKAAQPGS	GKGKVQFQGK 360
KTKFASDDEH	DEHDENGATG	PVKRAREETD	KEEPASKQQK 400
TENGAGDQ 408			

Pool	Sequence	Pool	Sequence
1	^{4}NGDNEKMAALEAKIC^{18}	8	^{1}MAENGDNEKMAALEA^{15}
	^{10}MAALEAKICHQIEYY^{24}		^{7}NEKMAALEAKICHQI^{21}
	^{16}KICHQIEYYFGDFNL^{30}		^{13}LEAKICHQIEYYFGD^{27}
	^{22}EYYFGDFNLPRDKFL^{36}		^{19}HQIEYYFGDFNLPRD^{33}
	^{28}FNLPRDKFLKEQIKL^{42}		^{25}FGDFNLPRDKFLKEQ^{39}
	^{34}KFLKEQIKLDEGWVP^{48}		^{31}PRDKFLKEQIKLDEG^{45}
	^{40}IKLDEGWVPLEIMIK^{54}		^{37}KEQIKLDEGWVPLEI^{51}
	^{46}WVPLEIMIKFNRLNR^{60}		^{43}DEGWVPLEIMIKFNR^{57}
	^{52}MIKFNRLNRLTTDFN^{66}		^{49}LEIMIKFNRLNRLTT^{63}
	^{58}LNRLTTDFNVIVEAL^{72}		^{55}FNRLNRLTTDFNVIV^{69}
2	^{64}DFNVIVEALSKSKAE^{78}	9	^{61}LTTDFNVIVEALSKS^{75}
	^{70}EALSKSKAELMEISE^{84}		^{67}VIVEALSKSKAELME^{81}
	^{76}KAELMEISEDKTKIR^{90}		^{73}SKSKAELMEISEDKT^{87}
	^{82}ISEDKTKIRRSPSKP^{96}		^{79}LMEISEDKTKIRRSP^{93}
	^{88}KIRRSPSKPLPEVTD^{102}		^{85}DKTKIRRSPSKPLPE^{99}
	^{94}SKPLPEVTDEYKNDV^{108}		^{91}RSPSKPLPEVTDEYK^{105}
	^{100}VTDEYKNDVKNRSVY^{114}		^{97}LPEVTDEYKNDVKNR^{111}
	^{106}NDVKNRSVYIKGFPT^{120}		^{103}EYKNDVKNRSVYIKG^{117}
	^{112}SVYIKGFPTDATLDD^{126}		^{109}KNRSVYIKGFPTDAT^{123}
	^{118}FPTDATLDDIKEWLE^{132}		^{115}IKGFPTDATLDDIKE^{129}
3	^{124}LDDIKEWLEDKGQVL^{138}	10	^{121}DATLDDIKEWLEDKG^{135}
	^{130}WLEDKGQVLNIQMRR^{144}		^{127}IKEWLEDKGQVLNIQ^{141}
	^{136}QVLNIQMRRTLHKAF^{150}		^{133}DKGQVLNIQMRRTLH^{147}
	^{142}MRRTLHKAFKGSIFV^{156}		^{139}NIQMRRTLHKAFKGS^{153}
	^{148}KAFKGSIFVVFDSIE^{162}		^{145}TLHKAFKGSIFVVFD^{159}
	^{154}IFVVFDSIESAKKFV^{168}		^{151}KGSIFVVFDSIESAK^{165}
	^{160}SIESAKKFVETPGQK^{174}		^{157}VFDSIESAKKFVETP^{171}
	^{166}KFVETPGQKYKETDL^{180}		^{163}SAKKFVETPGQKYKE^{177}
	^{172}GQKYKETDLLILFKD^{186}		^{169}ETPGQKYKETDLLIL^{183}
	^{178}TDLLILFKDDYFAKK^{192}		^{175}YKETDLLILFKDDYF^{189}

Pool	Sequence	Pool	Sequence
4	^{184}FKDDYFAKKNEERKQ^{198}	11	^{181}LILFKDDYFAKKNEE^{195}
	^{190}AKKNEERKQNKVEAK^{204}		^{187}DYFAKKNEERKQNKV^{201}
	^{196}RKQNKVEAKLRAKQE^{210}		^{193}NEERKQNKVEAKLRA^{207}
	^{202}EAKLRAKQEQEAKQK^{216}		^{199}NKVEAKLRAKQEQEA^{213}
	^{208}KQEQEAKQKLEEDAE^{222}		^{205}LRAKQEQEAKQKLEE^{219}
	^{214}KQKLEEDAEMKSLEE^{228}		^{211}QEAKQKLEEDAEMKS^{225}
	^{220}DAEMKSLEEKIGCLI^{234}		^{217}LEEDAEMKSLEEKIG^{231}
	^{226}LEEKIGCLIKFSGDL^{240}		^{223}MKSLEEKIGCLIKFS^{237}
	^{232}CLIKFSGDLDDQTCR^{246}		^{229}KIGCLIKFSGDLDDQ^{243}
	^{238}GDLDDQTCREDLHIL^{252}		^{235}KFSGDLDDQTCREDL^{249}
5	^{244}TCREDLHILFSNHGE^{258}	12	^{241}DDQTCREDLHILFSN^{255}
	^{250}HILFSNHGEIKWIDF^{264}		^{247}EDLHILFSNHGEIKW^{261}
	^{256}HGEIKWIDFVRGAKE^{270}		^{253}FSNHGEIKWIDFVRG^{267}
	^{262}IDFVRGAKEGIILFK^{276}		^{259}IKWIDFVRGAKEGII^{273}
	^{268}AKEGIILFKEKAKEA^{282}		^{265}VRGAKEGIILFKEKA^{279}
	^{274}LFKEKAKEALGKAKD^{288}		^{271}GIILFKEKAKEALGK^{285}
	^{280}KEALGKAKDANNGNL^{294}		^{277}EKAKEALGKAKDANN^{291}
	^{286}AKDANNGNLQLRNKE^{300}		^{283}LGKAKDANNGNLQLR^{297}
	^{292}GNLQLRNKEVTWEVL^{306}		^{289}ANNGNLQLRNKEVTW^{303}
	^{298}NKEVTWEVLEGEVEK^{312}		^{295}QLRNKEVTWEVLEGE^{309}
6	^{304}EVLEGEVEKEALKKI^{318}	13	^{301}VTWEVLEGEVEKEAL^{315}
	^{310}VEKEALKKIIEDQQE^{324}		^{307}EGEVEKEALKKIIED^{321}
	^{316}KKIIEDQQESLNKWK^{330}		^{313}EALKKIIEDQQESLN^{327}
	^{322}QQESLNKWKSKGRRF^{336}		^{319}IEDQQESLNKWKSKG^{333}
	^{328}KWKSKGRRFKGKGKG^{342}		^{325}SLNKWKSKGRRFKGK^{339}
	^{334}RRFKGKGKGNKAAQP^{348}		^{331}SKGRRFKGKGKGNKA^{345}
	^{340}GKGNKAAQPGSGKGK^{354}		^{337}KGKGKGNKAAQPGSG^{351}
	^{346}AQPGSGKGKVQFQGK^{360}		^{343}NKAAQPGSGKGKVQF^{357}
	^{352}KGKVQFQGKKTKFAS^{366}		^{349}GSGKGKVQFQGKKTK^{363}
	^{358}QGKKTKFASDDEHDE^{372}		^{355}VQFQGKKTKFASDDE^{369}

Pool	Sequence	Pool	Sequence
7	^{364}FASDDEHDEHDENGA^{378}	14	^{361}KTKFASDDEHDEHDE^{375}
	^{370}HDEHDENGATGPVKR^{384}		^{367}DDEHDEHDENGATGP^{381}
	^{376}NGATGPVKRAREETD^{390}		^{373}HDENGATGPVKRARE^{387}
	^{382}VKRAREETDKEEPAS^{396}		^{379}TGPVKRAREETDKEE^{393}
	^{388}ETDKEEPASKQQKTE^{402}		^{385}AREETDKEEPASKQQ^{399}
	^{394}PASKQQKTENGAGDQ^{408}		^{391}KEEPASKQQKTENGA^{405}

Appendix B3

Amino acid sequence of the La synthetic peptides and the 14 pools generated which were used for scanning the entire sequence in proliferation assays.

Appendix C1

Proliferative response by HLA DR3 DQ2 healthy individuals

Tritiated thymidine incorporation values (CPM) for healthy individuals with the HLA DR3 DQ2 haplotype. CPM values are initially given for the proliferative response to the peptide pools 1 - 14, tetanus toxoid peptide (TT) and HSP65 peptide (Hsp). Following this data are the CPM values for the proliferative response to individual peptides taken from pools 1 and 8 (amino acid range 1 - 72) and pools 3 and 10 (amino acid range 121 - 192) . Background proliferation levels are given as medium (med).

BW											Ave	SD
1	1923	2219	2149	1335	1992	999	1263	1099	4864	1365	1922	1126
2	2027	894	841	1500	580	420	862	579	962	1839	1050	551
3	1027	1241	1765	794	1096	1198	681	1144	1018	1198	1116	291
4	2126	522	2708	1336	711	1122	986	1416	731	942	1260	683
5	2164	10428	12047	15379	2125	13132	3698	14133	7789	7374	8827	4959
6	879	1216	1700	2309	1212	1235	1239	1630	1357	1310	1409	390
7	1507	754	861	584	1008	932	1225	977	1143	1057	1005	256
8	16977	12273	16363	2192	1615	15596	8605	2072	3829	8632	8815	6215
9	950	1200	1648	741	1266	1595	981	562	675	1174	1079	368
10	2658	1410	2018	2389	2097	2528	2766	2022	2055	1736	2168	421
11	1787	449	720	852	681	697	568	622	1006	776	816	374
12	1176	968	927	531	671	663	1032	1010	762	7373	1511	2069
13	528	574	1186	543	929	379	518	454	624	598	633	242
14	736	1070	1463	1382	1524	873	1882	1352	964	1025	1227	352
ttpep	1366	631	928	591	712	728	1090	910	929	2201	1009	479
HSP	5214	5792	1483	3239	1680	5482	3860	4790	2718	3438	3770	1537
TT	27382	26356	24897	28505	24349	27201	25765	22364	23280	27019	25712	1960
Rabies	1195	2049	3886	1957	2794						2376	1016
Med	754	896	997	1200	612	706	531	1002	834	685	822	205

JF*													Ave	SD
1	654	598	700	963	485	770	838	1507	1036	567	513	1313	804	352	793	323
2	523	460	718	496	354	610	446	528	515	421	513	486	556	633	518	92
3	943	562	553	424	414	424	367	414	347	427	597	869	462	523	523	179
4	422	376	551	359	503	620	592	491	442	438	371	651	476	450	482	93
5	825	412	364	559	463	271	389	498	538	542	674	782	502	465	535	138
6	399	436	553	318	661	576	470	437	466	551	607	386	542	482	492	95
7	429	554	672	619	573	566	435	637	578	469	518	568	693	658	569	84
8	1010	1195	1005	558	687	466	1617	2232	1099	1465	669	1222	1102	502	1059	489
9	581	675	500	517	443	592	650	448	859	463	723	554	466	512	570	121
10	1718	1728	1858	1091	484	674	462	1214	587	1770	663	1348	1058	640	1092	520
11	491	699	411	466	682	501	512	678	550	592	421	458	430	639	538	103
12	531	529	610	486	513	572	488	524	608	596	445	427	548	483	526	57
13	506	524	444	435	488	589	421	519	450	485	599	433	441	562	493	60
14	550	455	501	464	742	474	570	514	476	578	571	1203	516	556	584	192
ttpep	551	542	623	536	537	461	529	415	516	495	529	485	543	489	518	48
HSP	2612	1565	2540	1564	4000	3646	2073	2523	1514	1486	1332	3018	1989	1193	2218	877
TT	50320	45434	41333	48632	44738	40605	34363	41071							43312	5074
Rabies	2018	5800	1610	4453	1799	3949	2170	3989							3224	1533
Med	691	415	679	560	604	475	592	580	577	416	637	537	572	499	560	85

PK											Ave	SD
1	703	599	794	708	619	771	906	740	549	681	707	104
2	467	412	1099	300	873	485	503	839	579	864	642	258
3	945	692	564	543	930	361	701	815	953	588	709	200
4	344	816	469	335	372	666	589	492	420	459	496	154
5	3068	3893	656	2647	1452	884	872	1360	3753	1492	2008	1225
6	509	678	353	352	407	703	399	727	532	584	524	145
7	678	727	248	625	435	673	322	622	524	849	570	187
8	852	692	506	482	520	444	682	692	520	458	585	135
9	461	976	331	421	479	350	854	639	690	1266	647	305
10	951	938	6295	1850	615	1132	925	1132	801	465	1510	1722
11	1305	1047	609	427	596	885	644	790	621	1060	798	272
12	706	605	538	634	407	674	688	481	294	745	577	145
13	493	417	582	561	470	746	583	406	600	636	549	105
14	556	347	645	452	678	562	489	452	1052	566	580	192
ttpep	475	387	540	329	395	855	587	852	503	602	553	181
HSP												
TT	1219	1565	1384	1487							1414	149
Rabies	538	945	842	634	599	691	804				722	146
Med	579	505	422	399	378	332	392	526	416	462	441	76

HU													Ave	SD
1	4185	865	890	475	1179	446	418	2559	740						1306	1264
2	564	409	364	393	397	824	397	736	457						505	168
3	1579	1580	779	842	595	1072	687	615	490						915	412
4	332	396	364	324	725	337	461	485	340						418	129
5	484	423	784	852	1000	348	372	525	452						582	235
6	567	502	451	465	403	307	442	445	401						443	72
7	389	383	344	408	411	511	597	475	538						451	84
8	480	479	499	472	828	1092	1640	3006	965						1051	831
9	600	537	689	489	382	922	423	877	769						632	195
10	390	474	531	386	442	535	449	513	411						459	58
11	658	456	350	369	438	486	384	336	432						434	98
12	506	477	493	523	426	628	461	948	476						549	160
13	957	473	540	381	364	385	472	427	304						478	193
14	542	368	424	495	400	348	359	521	456						435	73
ttpep	460	380	438	443	440	325	412	413	562						430	64
HSP	3959	3642	3114	1849	2228	5388	3658	2588	2973						3267	1057
TT	77046	62743	71275												70355	7196
Rabies	1710	5526	2602												3279	1996
Med	447	471	477	474	451	404	430	410	432						444	27

PS												Ave	SD
1	488	454	855	599	495							578	164
2	421	461	460	414	441							440	22
3	669	484	624	592	527							579	74
4	839	378	658	792	574							648	184
5	2575	610	970	1385	844							1277	778
6	1569	407	297	758	499							706	512
7	781	561	489	510	496							567	123
8	5684	1556	1530	2923	2026							2744	1738
9	1534	715	504	918	573							849	414
10	471	355	458	461	357							420	59
11	615	581	545	613	502							571	48
12	584	679	547	481	515							561	76
13	491	409	435	445	441							444	30
14	576	450	561	462	568							523	62
ttpep	512	461	488	587	506							511	47
HSP	2449	1299	1066	1271	1625							1542	545
TT	27511	19908	22958	24375	19758	25329	21987	19959	27064	24833		23368	2916
Rabies	693	1467	815	906	709							918	319
Med	543	534	661	579	476							559	68

PB*																					Ave	SD
1	1298	1785	3412	1369	4394	1653															2318	1279
2	614	1474	1081	686	616	612															847	357
3	1089	937	4114	1432	1853	1016															1740	1211
4	527	1534	587	672	807	736															810	368
5	1579	2072	1166	1788	1896	2106															1768	353
6	571	1963	570	490	632	735															827	563
7	755	1112	534	670	610	567															708	213
8	1600	1536	1084	1118	1259	1342															1323	212
9	859	1989	919	1215	752	822															1093	467
10	818	830	1082	615	624	729															783	173
11	712	549	429	629	527	849															616	149
12	499	542	664	693	700	907															668	144
13	1160	574	693	430	500	532															648	266
14	545	628	673	706	705	680															656	61
ttpep	744	768	654	845	616	885															752	105
HSP	2496	1876	2648	2463	2101	1917															2250	328
TT	45732	44073	53684	49725	50173																48677	3817
Rabies	2796	1403	3590	968	1536	1943															2039	979
Med	805	638	732	635	697	706															702	63

JM*											Ave	SD
1	704	915	870	1090	863	985	1289	6136	2803	2564	1822	1685
2	699	591	611	534	646	695	655	1016	788	624	686	135
3	855	1023	645	687	1329	1099	3781	1350	2077	1684	1453	931
4	1270	1418	739	1060	1010	555	757	612	608	646	868	304
5	746	1232	912	735	752	2923	1653	6878	3818	2684	2233	1958
6	528	620	475	516	547	511	596	506	671	468	544	66
7	538	677	498	519	576	531	505	596	611	568	562	56
8	2065	1930	3263	1740	1648	7447	4899	2662	5003	3470	3413	1872
9	474	607	684	760	668	475	565	1062	700	698	669	168
10	1434	665	635	511	741	1436	1826	1764	1675	1578	1226	524
11	504	655	539	569	549	1302	649	611	854	765	700	237
12	535	643	678	588	474	2562	2264	1395	2074	1874	1309	820
13	459	456	616	573	525	597	496	592	510	625	545	64
14	481	546	529	533	469	601	563	558	652	515	545	54
ttpep	530	418	419	498	526	958	658	538	718	734	600	168
HSP	865	765	735	649	624	568	1139	481	696	992	751	199
TT	19600	24363	22581								22181	2407
Rabies	2584	1054	874	1925	1174	958					1428	681
Med	562	453	645	598	768	640	632	444	642	559	594	96

DP*																					Ave	SD
1	884	871	558	750	789	806	600	879	820	546	537	713	729	535	716	135						
2	559	540	511	544	574	498	591	587	651	438	532	517	520	577	546	51						
3	652	630	545	571	590	824	732	587	732	482	487	457	607	613	608	104						
4	907	1312	902	852	807	705	808	1232	466	512	920	965	941	413	839	258						
5	557	1062	567	584	542	545	660	473	729	577	467	457	552	590	597	152						
6	473	562	434	605	687	528	440	550	511	469	579	596	444	593	533	76						
7	340	514	450	538	564	774	608	592	1113	529	590	460	448	423	567	188						
8	1902	1566	1523	1097	1782	1134	934	1128	1085	1001	1069	1264	1290	1522	1307	302						
9	890	778	561	455	425	522	531	489	418	575	463	412	526	655	550	140						
10	612	836	952	876	542	793	654	718	530	565	747	650	873	537	706	143						
11	453	483	575	498	405	518	587	596	652	534	466	612	465	505	525	71						
12	422	488	482	588	562	511	515	431	569	410	437	342	455	495	479	68						
13	440	553	426	570	679	484	554	546	455	670	468	558	434	489	523	81						
14	516	424	663	414	360	436	481	501	510	496	447	568	451	462	481	73						
ttpep	534	411	466	491	525	546	602	587	477	463	587	673	522	541	530	68						
HSP	876	947	978	978	866	1120	1246	979	1472	824	798	1014	1514	1382	1071	240						
TT	9110	7005	8098	9402	8110	7705	8340								8253	813						
Rabies	995	1325	990	1104	1752	1259	836								1180	302						
Med	636	433	615	658	581	579	448	553	579	467	549	576	564	532	555	67						

HU															Ave	SD
1-15	489	482	536	601	527										527	48
4-18	533	709	767	565	644										644	97
7-21	514	540	581	617	563										563	39
10-24	503	419	639	395	489										489	95
13-27	442	609	445	395	473										473	81
16-30	642	866	397	327	558										558	213
19-33	456	401	542	437	959										559	230
22-36	590	631	967	410	650										650	201
25-39	340	982	515	431	567										567	247
28-42	322	642	323	557	461										461	142
31-45	516	413	398	568	473										474	71
34-48	661	457	398	262	445										445	144
37-50	307	396	289	394	346										346	49
40-54	490	479	480	283	433										433	87
43-57	494	504	459	618	519										519	60
46-60	408	567	454	353	446										446	79
49-63	349	550	535	419	463										463	83
52-66	448	519	527	348	461										461	72
55-69	335	459	615	544	488										488	104
58-72	452	558	463	411	471										471	54
TT	77046	62743	71275												70355	7196
Rabies	1710	5526	2602												3279	1996
Med	447	471	477	474	451	404	430	410	432						444	27

PS											Ave	SD
1-15	530	560	696	637	567	642	605	578	542		595	54
7-21	738	622	702	583	507	571	559	666	542		610	78
13-27	1348	918	779	941	972	870	1188	1036	812		985	183
19-33	655	605	606	617	632	566	562	724	605		619	49
25-39	383	377	411	493	758	335	304	564	665		477	156
31-45	588	364	460	500	537	372	373	400	513		456	83
37-50	417	373	598	464	466	538	426	550	450		476	72
43-57	433	642	642	539	640	483	584	803	470		582	115
49-63	2380	2633	3377	2793	3115	3565	2642	4614	2810		3103	681
55-69	781	1209	965	810	1016	763	1051	1048	1348		999	197
TT	27511	19908	22958	24375	19758	25329	21987	19959	27064	24833	23368	2916
Rabies	693	1467	815	906	709						918	319
Med	543	534	661	579	476						559	68

JF															Ave	SD	JM										Ave	SD
1-15	514	682	684	1362	477	508									704	335	1-15	785	750	817	782	634	610	572	957	878		754	128
4-18	649	642	630	832	643	636									672	79	4-18	598	589	599	473	1585	553	77	378	490		594	407
7-21	895	551	1196	901	1067	861									912	218	7-21	839	841	1372	836	653	679	1259	667	698		872	265
10-24	862	933	1141	862	958	1091									974	117	10-24	666	678	630	678	604	936	680	1162	686		747	182
13-27	843	689	696	792	708	999									788	120	13-27	911	1277	1057	1123	857	970	1099	1599	784		1075	247
16-30	894	823	564	646	690	613									705	128	16-30	580	759	655	841	545	625	659	706	788		684	98
19-33	1148	837	1111	1041	1114	3130									1397	856	19-33	671	771	833	706	900	658	762	907	639		761	102
22-36	1308	827	3209	800	741	1174									1343	942	22-36	656	502	723	5352	792	721	784	464	863		1206	1560
25-39	648	358	360	541	1483	632									670	418	25-39	825	1009	686	875	1213	940	874	1012	870		923	147
28-42	718	859	918	891	943	773									850	88	28-42	59	554	470	1012	384	427	525	662	466		507	252
31-45	766	659	534	449	482	404									549	138	31-45	622	809	860	718	1206	470	2040	1002	725		939	464
34-48	853	622	744	868	809	824									787	91	34-48	412	481	592	615	608	599	751	550	576		576	94
37-50	881	3135	1798	781	555	1019									1362	967	37-50	820	935	761	871	853	1198	1034	860	822		906	134
40-54	783	1439	935	1039	763	1176									1022	257	40-54	657	1918	482	590	823	508	508	5938	634		1340	1782
43-57	808	540	1149	1118	927	1070									935	232	43-57	650	938	921	811	3294	1052	1199	1033	861		1195	802
46-60	919	614	2024	589	650	1705									1084	625	46-60	460	623	631	429	492	388	463	357	496		482	94
49-63	4064	1328	2415	1323	1477	1354									1994	1098	49-63	2897	2102	2530	2313	2448	2222	3354	2146	1986		2444	436
52-66	1114	1618	1354	754	1324	1326									1248	291	52-66	485	7263	779	958	658	852	926	644	651		1468	2178
55-69	898	648	1164	776	654	774									819	193	55-69	971	1290	1164	880	681	1058	1074	1371	836		1036	221
58-72	1106	796	1191	1236	1154	1147									1105	158	58-72	729	1072	528	459	488	612	571	582	666		634	184
121-135	1228	642	1165	1171	884	922									1002	226	121-135	1140	1452	1362	1446	1507	919	894	682	685		1121	335
124-138	1249	924	1016	645	949	1040									971	197	124-138	544	737	558	482	421	636	766	687	586		602	115
127-141	989	1038	1029	896	610	963									921	161	127-141	612	699	1055	51	630	1128	620	780	733		701	308
130-144	1263	713	793	870	999	730									895	209	130-144	839	611	654	459	573	630	548	584	603		611	102
133-147	1119	965	1160	899	1334	776									1042	201	133-147	512	822	562	652	668	560	883	702	497		651	135
136-150	1193	1108	820	824	1033	923									983	153	136-150	462	753	511	560	601	563	599	1184	592		647	216
139-153	1233	1034	1244	909	839	1364									1104	209	139-153	934	596	965	590	882	861	648	656	694		758	151
142-156	1043	1173	843	865	914	738									929	155	142-156	720	895	626	661	470	850	420	660	564		652	157
145-159	964	564	1045	1212	860	982									938	217	145-159	865	1181	634	855	615	660	925	736	669		793	184
148-162	924	506	1079	823	660	821									802	200	148-162	547	660	763	855	580	640	476	904	939		707	166
151-165	1193	961	813	1182	715	706									928	221	151-165	613	669	904	718	944	1293	721	966	479		812	242
154-168	495	413	671	479	452	448									493	92	154-168	912	940	879	893	558	496	455	790	952		764	203
157-171	1354	993	988	880	643	813									945	238	157-171	634	724	806	552	442	651	584	557	749		633	114
160-174	888	715	1015	676	525	791									768	171	160-174	684	583	520	501	420	467	421	506	620		525	89
163-177	710	1224	976	808	883	815									903	181	163-177	757	669	882	1428	701	753	889	1274	676		892	275
166-180	778	981	880	979	891	659									861	124	166-180	365	605	385	420	510	490	348	508	364		444	88
169-183	980	1671	1620	1048	748	952									1170	382	169-183	1132	909	523	619	730	808	557	477	717		719	209
172-186	1221	860	845	1067	759	864									936	173	172-186	819	813	1172	673	630	781	868	800	699		806	158
175-189	431	443	1016	487	501	536									569	222	175-189	776	726	967	522	791	869	1257	975	927		868	203
178-192	590	618	1241	691	691	607									740	250	178-192	891	889	654	757	790	538	610	812	793		748	123
TT	50320	45434	41333	48632	44738	40605	34363	41071							43312	5074	TT	20715	26645	21976	22345							22920	2579
Rabies	2018	5800	1610	4453	1799	3949	2170	3989							3224	1533	Rabies	769	1068	1924	845	978	1765	835				1169	474
Med	691	415	679	560	604	475	592	580	577	416	637	537	572	499	560	85	Med	562	453	645	598	668	473	483	536	638		562	81

PB																Ave	SD	DP											Ave	SD
1-15	549	558	521	492	463	568	574	783								564	97	1-15	562	459	435	519	455	457	657				506	80
4-18	607	643	432	747	607	531										594	106	4-18	418	506	540	472	532	492	410	416			473	53
7-21	508	515	398	588	412	472	578	693								520	98	7-21	507	495	407	436	540	442	480				472	46
10-24	510	745	581	525	590	607										593	84	10-24	516	457	592	555	518	430	518	521			513	51
13-27	495	474	633	417	393	478	606	503								500	83	13-27	514	548	477	418	463	424	443				470	48
16-30	643	1792	647	459	885	611										840	486	16-30	495	489	481	518	473	477	519	485			492	18
19-33	494	524	455	436	543	512	481	516								495	36	19-33	483	537	471	488	461	444	503				484	30
22-36	785	852	471	584	673	476										640	159	22-36	544	536	538	475	434	475	487	509			500	39
25-39	585	480	482	527	456	501	453	529								502	44	25-39	494	482	508	888	559	436	503				553	152
28-42	456	533	515	533	509	536										514	30	28-42	537	518	443	478	509	506	452	443			486	37
31-45	480	502	475	556	502	507	538	414								497	43	31-45	459	509	492	490	447	488	559				492	36
34-48	526	588	667	593	593	558										588	47	34-48	497	489	554	494	546	476	513	501			509	28
37-50	542	518	522	502	451	546	425	455								495	46	37-50	512	489	469	1554	734	427	498				669	403
40-54	529	716	643	587	619	680										629	67	40-54	444	417	495	496	504	517	512	495			485	35
43-57	560	446	465	423	522	567	428	476								486	57	43-57	467	487	466	449	475	480	624				493	59
46-60	673	627	518	624	610	666										620	56	46-60	446	476	491	472	459	480	446	453			465	17
49-63	1818	1253	1607	1516	2561	1377	1499	1844								1684	407	49-63	1071	765	913	1538	1332	1411	1130				1166	277
52-66	738	823	513	470	636	571										625	135	52-66	468	633	509	483	529	604	583	692			563	78
55-69	751	551	422	579	518	564	615	454								557	101	55-69	552	687	450	540	457	463	414				509	93
58-72	717	603	694	610	656	658										656	45	58-72	811	732	905	1165	888	494	555	489			755	236
121-135	608	590	536	578	581											579	27	121-135	790	354	468	534	365	403	532				492	151
124-138	572	538	578	562	542											558	18	124-138	460	675	497	402	335	412	488				467	107
127-141	755	500	514	556	541											573	104	127-141	640	390	308	434	439	326	478				431	111
130-144	636	578	616	610	564											601	29	130-144	572	861	534	473	316	424	319				500	187
133-147	706	744	623	691	668											686	45	133-147	609	330	394	423	567	406	876				515	187
136-150	494	544	703	580	571											579	77	136-150	443	607	427	362	379	342	478				434	90
139-153	688	553	474	571	546											566	77	139-153	542	424	408	432	691	424	538				494	103
142-156	633	588	626	616	601											613	18	142-156	551	434	459	342	325	511	327				421	92
145-159	607	521	575	568	570											568	31	145-159	453	414	368	423	363	383	404				401	32
148-162	681	632	643	652	673											656	21	148-162	482	405	537	495	400	498	548				481	58
151-165	503	1186	522	737	612											712	281	151-165	1012	2002	368	543	443	396	579				763	588
154-168	571	547	1733	850	569											854	507	154-168	422	428	644	450	456	461	942				543	192
157-171	572	1570	561	901	602											841	431	157-171	520	372	630	401	547	333	331				448	118
160-174	544	520	519	528	533											529	10	160-174	478	460	544	434	426	507	486				476	41
163-177	768	512	628	636	595											628	92	163-177	593	355	391	369	474	456	604				463	102
166-180	624	547	591	587	571											584	28	166-180	660	598	476	512	524	486	512				538	67
169-183	882	675	829	795	754											787	78	169-183	862	445	310	743	879	511	483				605	222
172-186	1396	625	820	948	696											897	305	172-186	1238	1229	1366	1189	916	718	1209				1123	224
175-189	430	552	500	427	498											481	53	175-189	428	467	437	404	487	436	465				446	28
178-192	631	560	459	483	544											536	68	178-192	545	547	426	496	556	508	476				508	47
TT	45732	44073	53684	49725	50173											48677	3817	TT	4089	4431	4832	4784							4534	346
Rabies	2796	1403	3590	968	1536	1943										2039	979	Rabies	1488	786	1168	481	647	597					861	388
Med	805	638	732	635	697	706										702	63	Med	542	502	520	472	531	461	524				507	31

Appendix C2

Proliferative response by HLA DR3 DQ2 patients

Tritiated thymidine incorporation values (CPM) for patients with SLE with the HLA DR3 DQ2 haplotype. CPM values are initially given for the proliferative response to the peptide pools 1 - 14, tetanus toxoid peptide (TT) and HSP65 peptide (Hsp). Following this data are the CPM values for the proliferative response to individual peptides taken from pools 1 and 8 (amino acid range 1 - 72) and pools 3 and 10 (amino acid range 121 - 192) . Background proliferation levels are given as medium (med).

BM											Ave	SD
1	398	193	251	386	370	423	260	468	438	487	367	99
2	265	124	269	238	209	299	237	228	294	381	254	67
3	744	584	557	684	595	743	747	711	603	582	655	78
4	244	709	218	257	310	377	247	174	200	225	296	156
5	226	138	237	171	274	146	198	229	256	295	217	53
6	294	274	232	266	445	435	380	400	449	533	371	99
7	353	117	153	113	133	313	209	173	197	344	211	93
8	118	390	111	106	110	132	126	116	372	267	185	114
9	104	160	219	201	267	255	167	141	186	198	190	50
10	224	161	171	290	263	149	126	175	196	179	193	51
11	161	143	271	252	234	373	292	150	164	214	225	74
12	157	197	143	159	198	143	141	207	195	173	171	26
13	177	138	184	132	244	198	182	235	256	242	199	44
14	139	174	252	391	220	180	209	191	329	263	235	77
ttpep	248	115	250	139	131	124	166	201	230	185	179	52
HSP	220	204	156	165	127	106	152	277	253	387	205	84
TT	98	137	157	101	150	906					258	318
PPD												
Rabies	95	98	434	148	183	148	141	137	427		201	133
Med	231	189	180	185	236	178	104	212	182	165	186	37

JC											Ave	SD
1	612	542	571	676	586	668					609	54
2	399	323	304	226	305	315					312	55
3	481	334	410	380	422	392					403	49
4	385	269	262	261	272	328					296	50
5	303	291	320	275	266	327					297	24
6	214	214	283	206	224	268					235	32
7	1665	244	234	252	244	236					244	8
8	1446	852	959	1037	1021	958					1046	207
9	312	200	203	265	231	315					254	51
10	961	1104	822	853	924	973					940	100
11	264	341	264	266	289	267					282	30
12	314	284	210	278	255	280					270	35
13	234	271	322	282	244	265					270	31
14	301	205	270	260	268	274					263	32
ttpep	247	242	303	253	269	275					265	23
HSP	423	318	407	372	388	364					379	37
TT	170	254	242	244							327	58
PPD	33915	26236	25667	29417							28809	3783
Rabies	307	275	305	365							313	38
Med	317	322	363	280	297	335					319	29

GC											Ave	SD
1	1204	1265	1225	1253	1068	878					1149	151
2	357	178	264	155	306	226					248	77
3	549	399	583	721	255	326					472	175
4	207	292	251	298	209	247					251	39
5	256	398	318	237	285	331					304	58
6	317	409	440	361	325	338					365	49
7	294	333	329	244	254	240					282	42
8	231	346	774	690	236	643					487	243
9	225	209	259	344	435	284					293	84
10	727	573	1775	463	1320	831					948	502
11	256	534	301	232	497	344					361	126
12	317	266	352	749	324	778					464	234
13	207	267	293	339	212	296					269	52
14	629	302	287	200	204	385					335	160
ttpep	346	214	267	365	251	317					293	59
HSP	945	873	1070	964	1109	927					981	90
TT	4289	5497	5122								4969	618
Rabies	250	280	273	221	268	324					269	34
Med	336	203	251	325	197	205					253	63

JMA											Ave	SD
1	373	493	446	453	384						430	50
2	519	432	522	440	463						475	43
3	373	426	490	398	458						429	46
4	1665	1534	1011	1236	1187						1327	267
5	814	1012	954	836	995						922	92
6	718	640	596	598	624						635	50
7	497	504	504	544	482						506	23
8	501	443	507	467	478						479	26
9	326	497	361	382	369						387	65
10	430	330	606	452	475						459	99
11	518	432	394	437	446						446	45
12	814	677	873	798	862						805	78
13	517	517	400	416	443						459	55
14	467	304	452	379	364						393	67
ttpep	656	563	666	624	577						617	46
HSP	1428	905	1431	1368	347						1096	473
TT	2690	1902	2818								2470	496
Rabies	472	644	551	536	426						526	83
Med	296	491	411	432	359						398	74

HK												Ave	SD
1	795	726	979	1028	1389	982						983	232
2	522	206	205	804	427	266						405	233
3	1003	1043	604	1181	1450	966						1041	277
4	220	328	446	237	597	486						386	149
5	382	639	461	606	1470	646						701	392
6	229	383	319	273	221	373						299	70
7	253	243	223	290	341	388						290	64
8	1500	1758	1613	1582	2089	1523						1678	221
9	385	465	458	316	488	542						442	80
10	367	1043	610	754	606	868						708	235
11	298	256	277	224	303	272						272	29
12	334	613	523	411	416	505						467	99
13	265	275	231	211	244	247						246	23
14	286	376	257	209	280	384						299	69
ttpep	684	906	226	495	337	551						533	244
HSP	698	589	633	547	568	574						602	55
TT	532	410	255	381	309							377	106
PPD	2457	2075	3160	2918	3064							2735	457
Rabies	328	372	408	436	360							381	42
Med	432	410	255	298	264	365						337	76

BF												Ave	SD
1	4494	2119	2479	3401	2327	2579						2900	896
2	473	296	308	286	344	376						347	70
3	344	464	4679	2143	6488	2674						2799	2411
4	284	589	497	467	400	427						444	102
5	268	246	236	290	524	347						319	108
6	198	205	278	431	322	316						292	86
7	240	202	253	609	549	316						362	173
8	1493	1440	1367	1060	1070	1150						1263	193
9	294	257	392	570	563	424						417	131
10	241	276	246	307	397	332						300	59
11	295	558	326	505	461	426						428	102
12	240	338	252	540	468	379						370	119
13	272	217	373	390	310	305						311	64
14	235	455	231	456	496	343						369	117
ttpep	475	388	245	510	483	417						420	97
HSP	385	244	476	465	383	343						383	85
TT	700	1152	769	900	797	944						877	161
PPD	5572	5585	4582	6183	5728	4543						5366	660
Rabies	465	383	320	432	389	354						390	52
Med	246	335	356	305	296	276						303	40

AL												Ave	SD
1	652	710	682	500	594	466						601	99
2	232	362	389	232	220	265						283	73
3	2177	481	1515	588	542	590						982	702
4	398	452	535	350	271	231						373	113
5	3079	346	434	2308	271	346						1131	1236
6	355	200	259	250	369	273						284	65
7	267	238	237	241	320	235						256	33
8	1351	1264	988	945	1081	1238						1144	164
9	219	289	358	276	203	372						286	69
10	786	588	656	582	603	691						651	79
11	217	206	230	233	247	264						233	21
12	296	270	211	286	233	260						259	33
13	326	343	213	201	274	279						273	57
14	273	244	241	352	339	206						275	58
ttpep	279	391	240	292	270	251						287	54
HSP	855	216	297	224	368	313						379	240
TT	25155	35961	33319	26947	31296							30536	4461
Rabies	2395	543	619	984	548	1267						1059	715
Med	471	280	205	367	323	290						323	90

HCO												Ave	SD
1	987	305	244	4534	765	1095						1322	1612
2	348	270	210	231	339	205						267	63
3	639	273	284	312	392	566						411	156
4	223	241	307	269	271	213						254	35
5	310	205	248	233	318	459						296	91
6	318	206	250	308	265	320						278	46
7	417	498	361	442	344	277						390	79
8	616	825	225	427	331	792						536	247
9	251	398	296	335	242	276						300	59
10	250	384	284	302	271	364						309	53
11	279	379	227	364	321	330						317	56
12	322	245	257	252	325	312						285	38
13	202	386	269	252	354	220						281	74
14	338	281	267	279	467	388						337	78
ttpep	509	213	226	375	337	326						331	108
HSP	454	398	347	424	363	376						394	40
TT	1590	1491	1436	1462								1495	67
Rabies	1244	460	2055	395	455	945						926	648
Med	272	301	346	296	279	326						303	28

APO												Ave	SD
1	903	1069	874	1152	953	1065						1003	109
2	366	399	274	416	364	383						367	50
3	2343	1703	1357	1593	1235	1675						1651	386
4	565	303	317	251	340	352						355	109
5	1008	966	789	800	759	852						862	102
6	458	261	348	299	304	343						335	68
7	583	360	351	305	276	362						373	108
8	3178	2520	2646	2144	2762	2648						2650	336
9	587	349	389	324	350	368						394	97
10	1666	1525	1277	1285	1742	1428						1487	194
11	493	410	350	297	260	368						363	83
12	223	403	361	560	387	459						399	111
13	332	380	350	479	451	386						396	57
14	586	368	349	390	309	302						384	104
ttpep	305	334	384	412	339	382						359	40
HSP	459	409	436	431	493	553						464	52
TT	2679	3461	3052									3064	391
Rabies	645	925	538	761	594							693	154
Med	504	567	638	492	499	502						534	58

JP														Ave	SD
1	292	336	262	383	422	1798	1283	670	594					671	527
2	237	172	236	197	198	196	206	211	172					202	23
3	627	316	328	464	280	364	208	265	332					354	125
4	248	134	170	230	244	170	191	200	160					194	40
5	544	347	254	234	187	311	485	314	200					319	124
6	162	204	169	153	170	204	131	156	278					181	43
7	231	178	174	263	271	200	202	162	149					203	43
8	373	364	251	499	375	460	420	297	450					388	79
9	231	173	171	182	196	157	180	158	159					179	23
10	250	303	225	1658	267	322	205	226	330					421	466
11	319	366	238	427	307	312	304	362	398					337	57
12	321	209	216	298	291	208	272	306	203					258	49
13	193	165	135	159	177	159	138	134	173					159	20
14	203	176	200	198	191	214	180	147	126					182	29
ttpep	124	127	161	198	126	169	192	131	225					161	37
HSP	164	127	137	161	207	125	160	171	167					158	26
TT	4440	4194	3788	3807										4057	316
Rabies	274	634	260	207	658	258								382	206
Med	169	198	160	164	156	165	171	153	163					167	13

HJ												Ave	SD
1	1194	1334	1685	1231	1463	959	2639	689	614	1552	1487	1350	549
2	520	539	927	442	648	942	602	546	745	972	673	687	186
3	1725	1778	1821	2310	1944	6953	1073	2667	800	555	1218	2077	1737
4	829	381	572	372	648	270	955	621	404	266	812	557	238
5	2744	982	964	1062	1161	534	235	382	261	1378	1218	993	707
6	578	473	342	412	414	598	299	490	340	355	618	447	113
7	617	748	822	630	628	472	493	524	306	386	607	567	151
8	2847	1879	2267	2266	2057	1267	1007	8128	5411	2816	1692	2876	2096
9	1158	930	542	660	746	487	430	257	434	389	657	608	261
10	7167	5924	4769	6831	5575	12823	2026	1623	3099	1897	1621	4850	3393
11	1233	388	450	455	660	553	564	436	898	459	517	601	253
12	1260	420	658	2014	1359	747	1316	1416	1172	523	796	1062	477
13	440	265	332	445	543	691	557	460	1550	586	1297	651	403
14	515	445	365	621	536	566	738	656	738	487	726	581	126
ttpep	1098	1080	1508	662	769	6582	6628	4608	1504	960	917	2392	2350
Hep	1450	1573	1260	1195	1467	1867	1976	2121	2025	1756	2067	1705	333
TT	19004	20521	22603	21376	19580							20617	1432
PPD													
Rabies	704	634	1060	2007	658	2558						1270	818
Med	585	638	690	652	662	576	554	487	504	558	594	591	65

DP																Ave	SD
1	583	1208	914	6532	1190	1450	1345	1502	1722	1417	2340	1594	1816	1546		1797	1424
2	517	761	687	776	1121	809	818	802	1160	609	1357	1138	880	854		878	234
3	491	499	943	877	727	667	503	465	656	456	862	1209	646	743		696	219
4	459	514	671	398	458	383	579	536	587	659	401	657	349	740		528	124
5	1049	1009	1016	2540	5148	674	2186	1607	4065	918	2434	1367	2001	1383		1957	1283
6	1453	457	2932	973	3619	332	3745	506	1371	973	1036	535	1494	1234		1476	1137
7	806	1002	619	904	1283	817	969	490	1112	840	822	843	876	208		828	262
8	997	3485	2239	1392	3082	778	1115	1227	2045	3109	4841	2958	2272	1244		2199	1177
9	693	755	731	811	1316	910	828	541	1229	857	1082	1084	903	531		877	234
10	523	422	815	700	1316	1321	839	525	1400	466	1775	1391	899	516		922	438
11	493	530	490	638	403	477	339	417	352	349	862	336	340	578		472	148
12	740	1621	557	1896	482	1028	1063	2387	503	939	2729	1801	787	910		1246	720
13	418	665	739	774	408	333	494	378	643	791	558	704	1100	921		638	222
14	436	1038	562	740	979	603	704	601	862	1008	576	786	741	196		702	232
ttpep	736	672	578	1387	1202	793	978	574	951	819	1214	1560	955	322		910	341
Hep	618	821	7545	1332	8187	1037	1513	7284	3639	1852	2072	1491	3116	2858		3097	2630
TT	417	580	591	384	580											510	101
PPD	42176	51002	47716	51239	43415											47110	4200
Rabies	529	697	396	495	746	584	604	761	634	487						593	119
Med	680	520	572	338	510	766	689	503	384	694	464	321	537	307		520	149

PBR												Ave	SD		DW												Ave	SD
1	354	372	375	388	552	473						419	77		1	525	1062	794	896	723								800	200
2	335	469	342	397	280	378						367	64		2	282	328	305	277	307								300	21
3	368	335	387	349	422	402						377	33		3	1191	1002	1096	998	1067								1071	79
4	405	376	384	429	281	369						374	50		4	346	261	303	326	282								304	34
5	426	316	273	423	381	362						363	60		5	1252	1120	1186	1166	1236								1192	53
6	543	267	713	356	415	579						479	163		6	1818	380	1099	421	411								826	631
7	246	281	232	268	483	347						309	94		7	503	292	397	406	336								387	80
8	1956	753	410	952	465	1689						1038	645		8	3884	875	2380	1168	1492								1960	1214
9	335	438	310	220	251	365						320	79		9	377	233	305	267	328								302	55
10	490	370	308	323	413	362						378	67		10	612	581	596	605	587								596	13
11	320	381	362	359	319	335						346	25		11	362	300	331	350	333								335	23
12	319	433	338	350	334	346						353	40		12	1705	1812	1758	1734	1628								1727	68
13	513	368	456	530	619	473						493	84		13	589	362	476	398	468								459	87
14	451	254	314	370	293	346						338	69		14	267	280	274	368	327								303	43
ttpep	508	753	445	325	348	469						475	154		ttpep	365	325	302	336	298								325	27
HSP	600	489	798	687	644	695						652	104		HSP	309	260	285	277	301								287	19
TT	1665	1506	1699									1624	103		TT	6220	6631	6842										6564	316
Rabies	512	451	441	521	531	465						487	39		Rabies	336	304	325	295	364								325	27
Med	375	371	287	381	292	364						345	43		Med	282	378	330	356	300								329	40

GC								Ave	SD	JC								Ave	SD
1-15	303	318	444	222	337			325	80	1-15	359	306	291	386	270	283		316	46
4-18	225	267	232	268	254			249	20	4-18	405	613	551	481	527	562		523	72
7-21	273	296	328	266	290			291	24	7-21	395	324	319	354	275	296		327	43
10-24	290	277	311	258	297			287	20	10-24	399	424	363	494	393	419		415	44
13-27	247	292	295	268	306			282	24	13-27	508	427	532	338	422	450		446	69
16-30	289	294	262	276	251			274	18	16-30	405	431	496	545	392	370		440	67
19-33	334	275	304	271	317			300	27	19-33	420	351	311	492	338	329		374	69
22-36	305	797	426	368	299			439	207	22-36	268	456	347	309	354	364		350	63
25-39	272	299	301	357	277			301	34	25-39	188	223	183	204	224	201		204	17
28-42	211	301	234	264	252			252	34	28-42	327	302	283	347	327	316		317	22
31-45	230	277	280	262	243			258	22	31-45	290	326	299	423	261	318		320	56
34-48	291	304	295	268	264			284	18	34-48	298	223	225	282	270	267		261	31
37-50	287	295	294	267	309			290	15	37-50	433	429	255	618	299	401		406	127
40-54	284	289	310	257	260			280	22	40-54	277	240	218	278	228	250		248	25
43-57	330	276	291	366	324			317	35	43-57	364	341	301	293	367	331		333	31
46-60	335	309	1474	468				646	556	46-60	302	295	307	325	314	281		304	15
49-63	437	304	344	329	345			352	50	49-63	1959	1843	2194	1957	1729	1960		1940	155
52-66	282	273	297	287	350			298	30	52-66	360	297	598	351	397	349		392	106
55-69	333	306	297	345	276			311	28	55-69	459	371	334	432	261	290		358	78
58-72	253	351	319	244	297			293	45	58-72	287	324	244	288	291	273		284	26
121-135	251	230	330	292	276			276	38	121-135	524	831	329	1222	447			671	360
124-138	279	304	266	260	235			269	25	124-138	329	328	344	622	335			392	129
127-141	281	290	280	273	308			286	14	127-141	319	352	322	319	372			337	24
130-144	261	276	236	228	247			250	19	130-144	338	328	326	531	305			366	93
133-147	270	297	316	296	287			293	17	133-147	385	367	317	446	333			370	50
136-150	233	280	276	263	279			266	20	136-150	440	548	397	356	362			421	79
139-153	268	235	282	287	304			275	26	139-153	384	341	327	395	340			357	30
142-156	252	272	314	232	281			270	31	142-156	365	386	331	767	607			491	189
145-159	246	280	254	303	292			275	24	145-159	282	303	287	317	277			293	16
148-162	293	277	230	267	274			268	23	148-162	242	293	263	427	233			292	79
151-165	279	284	228	269	291			270	25	151-165	334	327	283	303	443			338	62
154-168	299	260	273	247	263			268	19	154-168	270	276	351	271	261			286	37
157-171	277	294	248	288	237			269	25	157-171	300	263	173	256	244			247	46
160-174	297	228	237	254	280			259	29	160-174	272	886	502	350	302			462	253
163-177	262	241	265	179	284			246	41	163-177	374	316	344	382	263			336	48
166-180	299	266	306	257	277			281	21	166-180	271	804	473	343	373			453	209
169-183	269	231	288	248	227			253	26	169-183	341	284	272	315	408			324	54
172-186	318	272	273	254	295			282	25	172-186	357	379	350	315	333			347	24
175-189	239	268	237	313	246			261	32	175-189	441	453	370	501	399			433	51
178-192	270	230	315	238	358			282	54	178-192	405	284	385	464	355			379	66
TT	3333	4152	3452					3646	442	TT	508	537	437	468				488	44
Rables	335	295	398	267	284			613	52	PPD	30043	26959	20444	26914				26090	4039
Med	294	349	265	259	273			288	37	Rables	386	520	435	606	405			470	92
										Med	316	357	309	395	372			350	37

JMA								Ave	SD		BF								Ave	SD
1-15	431	395	407	426	422			416	15		1-15	254	276	307	260	259	298		276	22
4-18	554	336	377	398	433			420	83		4-18	534	257	362	431	442			405	103
7-21	398	401	391	374	364			386	16		7-21	342	357	290	323	334	322		328	23
10-24	549	552	361	404	486			470	86		10-24	561	362	335	376	469			421	93
13-27	513	407	442	406	433			440	44		13-27	539	431	1155	635	916	320		666	314
16-30	532	558	424	470	507			498	53		16-30	368	457	412	429	397			413	33
19-33	392	344	360	343	350			358	20		19-33	334	321	337	317	332	293		322	16
22-36	589	760	762	534	636			656	102		22-36	401	265	384	372	346			354	53
25-39	298	318	326	362	331			327	23		25-39	367	392	394	342	374	338		368	24
28-42	363	486	297	392	334			374	72		28-42	388	323	502	364	437			403	69
31-45	303	290	300	307	284			297	10		31-45	286	348	364	292	291	262		307	40
34-48	433	526	496	457	453			473	37		34-48	427	389	211	342	292			332	85
37-50	325	383	344	323	357			346	25		37-50	392	332	553	350	358	265		375	97
40-54	653	480	384	425	460			480	103		40-54	344	425	393	411	372			389	32
43-57	363	433	391	377	409			395	27		43-57	431	416	408	395	402	461		419	24
46-60	608	446	448	504	527			507	67		46-60	539	373	340	362	429			409	80
49-63	558	617	664	815	740			679	101		49-63	2741	2079	2661	2386	2386	2063		2386	283
52-66	1149	1555	1161	1283	962			1222	219		52-66	554	274	396	308	418			390	109
55-69	375	319	362	392	356			361	27		55-69	306	234	262	270	252	304		271	29
58-72	586	485	470	476	510			505	48		58-72	416	287	276	359	345			337	57
121-135	421	442	326	396	373			392	45		121-135	262	281	293	273	340	295		291	27
124-138	313	359	311	452	459			379	73		124-138	358	242	248	239	365	298		292	58
127-141	389	397	387	391	425			398	16		127-141	278	287	299	235	275	257		272	23
130-144	395	353	361	303	453			373	56		130-144	329	242	214	228	354	271		273	57
133-147	338	350	340	342	376			349	16		133-147	315	245	267	272	301	318		286	29
136-150	398	402	358	420	392			394	23		136-150	221	237	301	368	233	316		279	58
139-153	348	350	374	324	391			357	26		139-153	349	309	215	293	241	380		298	63
142-156	317	361	406	355	326			353	35		142-156	280	220	271	397	220	390		296	79
145-159	220	364	454	346	427			362	91		145-159	275	357	329	323	250	264		300	43
148-162	356	335	332	319	389			346	27		148-162	253	287	346	270	296	242		282	37
151-165	327	284	291	301	416			324	54		151-165	250	249	390	377	289	242		300	67
154-168	458	476	431	540	406			462	51		154-168	376	280	308	321	252	244		297	49
157-171	332	408	333	325	425			365	48		157-171	267	254	299	323	353	331		304	38
160-174	389	392	477	310	384			390	59		160-174	219	263	286	312	275	239		266	33
163-177	294	356	352	467	402			374	64		163-177	258	306	288	377	275	348		309	46
166-180	393	358	443	349	362			381	38		166-180	239	282	236	370	346	239		285	59
169-183	407	955	365	539	491			551	236		169-183	287	254	357	310	258	291		293	38
172-186	590	400	229	381	417			403	128		172-186	279	285	275	323	232	271		278	29
175-189	413	324	338	358	458			378	56		175-189	292	289	268	316	273	335		296	26
178-192	414	427	414	452	424			426	16		178-192	263	275	302	294	233	256		270	25
TT	1530	1093	1169					1264	234		TT	907	885	837	875	764	790	852	844	52
Rabies	701	779	458	523	479			588	143		PPD	9060	8704	6530	5454	8801	7349	7513	7630	1330
Med	312	291	250	346	325			305	37		Rabies	603	596	373	493	382	369		469	111
											Med	319	302	256	319	249	372		303	46

JP								Ave	SD	AL								Ave	SD
1-15	222	201	160	164	144	140		172	33	1-15	272	305	237	238	307			272	34
4-18	141	122	206	207	210	147		172	40	4-18	279	236	297	247	256			263	25
7-21	166	135	174	130	113	168		148	25	7-21	259	311	280	350	315			303	35
10-24	126	146	151	178	136	143		147	18	10-24	220	314	306	247	279			273	40
13-27	168	144	181	178	144	153		161	17	13-27	399	328	268	265	279			308	57
16-30	148	154	144	149	142	141		146	5	16-30	384	298	306	256	346			318	49
19-33	171	135	106	186	129	165		149	30	19-33	255	223	306	428	283			299	79
22-36	342	393	214	176	192	253		262	87	22-36	206	429	216	267	272			278	89
25-39	157	151	135	133	138	178		149	17	25-39	308	429	346	339	320			348	48
28-42	149	156	142	210	151	182		165	26	28-42	325	428	241	336	332			332	66
31-45	162	128	221	144	124	135		152	36	31-45	349	290	260	312	353			313	39
34-48	163	151	205	147	148	173		164	22	34-48	338	231	428	407	231			327	94
37-50	192	144	146	161	138	150		155	20	37-50	239	303	350	307	425			325	69
40-54	161	144	181	135	154	164		156	16	40-54	239	326	317	228	247			271	46
43-57	166	132	196	176	104	120		149	36	43-57	243	236	329	226	234			254	43
46-60	573	780	136	175	142	397		367	266	46-60	293	287	320	344	261			301	32
49-63	329	417	583	276	373	534		419	119	49-63	236	304	236	401	319			299	68
52-66	151	161	163	133	124	172		151	19	52-66	217	303	255	337	328			288	51
55-69	145	147	168	180	174	191		168	18	55-69	358	291	344	324	254			314	42
58-72	141	119	186	202	207	231		181	43	58-72	200	241	313	249	251			251	40
121-135	151	136	155	140	148	163		149	10	121-135	264	362	292	272	302			298	39
124-138	160	148	219	136	144	189		166	32	124-138	348	242	344	335	402			334	58
127-141	181	148	64	187	166	192		156	48	127-141	277	341	256	258	208			268	48
130-144	128	142	132	137	132	139		135	5	130-144	355	234	337	410	374			342	66
133-147	170	147	151	168	134	176		158	16	133-147	365	428	268	254	259			315	78
136-150	163	126	158	123	141	134		141	17	136-150	342	362	283	360	406			351	45
139-153	168	148	184	176	154	175		168	14	139-153	340	239	314	331	293			303	40
142-156	132	134	164	159	168	148		151	15	142-156	288	376	374	367	326			346	38
145-159	146	140	190	324	229	184		202	68	145-159	362	329	282	258	287			304	41
148-162	167	233	232	240	146	125		190	51	148-162	279	339	309	328	293			310	25
151-165	179	149	154	146	180	168		163	15	151-165	355	266	323	349	290			317	38
154-168	147	164	217	159	184	189		177	25	154-168	237	350	321	290	340			308	46
157-171	177	165	194	154	184	161		172	15	157-171	330	236	273	346	281			293	45
160-174	154	137	150	164	176	171		159	14	160-174	331	347	246	264	274			292	44
163-177	162	191	164	204	138	173		172	23	163-177	332	450	261	238	249			306	88
166-180	157	147	176	182	143	184		165	18	166-180	260	242	350	316	329			299	46
169-183	169	233	180	224	200	175		197	27	169-183	236	305	262	334	275			282	38
172-186	154	132	201	264	171	234		193	50	172-186	289	276	291	349	247			290	37
175-189	140	228	207	170	203	163		185	33	175-189	308	242	261	335	280			285	37
178-192	138	229	218	221	214	279		216	45	178-192	344	241	347	231	291			291	55
TT	5697	6034	5071	5963				5691	438	TT	25155	35961	33319	26947	31296			30536	4461
Rabies	249	229	311	268	172			246	51	Rabies	2395	543	619	984	548	1267		1059	715
Med	240	181	213	167	164	205	234	201	31	Med	471	280	205	367	323	290		323	90

APO								Ave	SD		HJ								Ave	SD
1-15	620	1066	658	1175	980			900	248		1-15	281	367	319	337	306			322	32
4-18	1166	737	873	669	834	973		875	177		4-18	272	367	262	296	283			296	42
7-21	1260	1050	907	955	1134			1061	141		7-21	442	437	380	452	402			423	30
10-24	1310	780	1024	522	1132	642		902	304		10-24	433	330	314	348	361			357	46
13-27	822	819	955	1123	893			922	125		13-27	814	406	317	589	529			531	190
16-30	1356	727	1079	1324	1174	1344		1167	242		16-30	636	415	334	580	472			487	122
19-33	939	1884	961	918	1214			1183	410		19-33	314	375	420	373	352			367	39
22-36	376	1076	320	314	550	481		520	288		22-36	428	358	306	368	351			362	44
25-39	538	1212	695	642	759			769	260		25-39	399	310	638	442	497			457	122
28-42	1008	915	1121	1117	1065	1050		1046	77		28-42	453	372	326	367	387			381	46
31-45	667	794	592	899	783			747	119		31-45	315	368	348	320	303			331	27
34-48	1019	842	1049	4050	1636	1993		1765	1201		34-48	316	322	287	424	393			348	57
37-50	601	1514	1095	967	1063			1048	326		37-50	328	290	340	342	358			332	26
40-54	3666	825	1071	828	968	2760		1686	1220		40-54	383	357	362	401	359			372	19
43-57	483	645	610	540	572			570	63		43-57	380	328	317	396	361			356	34
46-60	1041	1133	926	966	1068	981		1019	76		46-60	383	436	377	427	441			413	30
49-63	510	1014	744	902	941			822	201		49-63	2086	1906	1568	1971	2093			1925	215
52-66	1108	972	1079	769	936	945		968	121		52-66	466	342	411	423	430			414	45
55-69	876	1007	1058	960	957			972	67		55-69	411	377	347	384	379			380	23
58-72	1242	1352	1316	2024	1565	1497		1499	283		58-72	386	319	367	348	329			350	27
121-135	948	919	849	921	930			913	38		121-135	599	620	591	555	574			588	25
124-138	969	803	837	651	840			820	114		124-138	570	508	578	585	628			574	43
127-141	1093	751	1243	920	894			980	191		127-141	573	584	576	572	607			582	15
130-144	670	1204	1074	1068	952			994	202		130-144	506	504	593	434	552			518	60
133-147	1223	1112	987	1053	990			1073	98		133-147	638	578	667	586	592			612	39
136-150	1208	1103	1320	1547	1014			1238	207		136-150	409	431	492	477	501			462	40
139-153	1106	915	1087	984	1028			1024	78		139-153	656	562	568	585	636			601	42
142-156	612	916	712	662	860			752	130		142-156	448	502	600	517	471			508	58
145-159	1428	1386	2245	1572	2228			1772	430		145-159	657	512	537	642	574			584	64
148-162	1297	1176	1331	1513	1538			1371	153		148-162	454	441	516	470	492			475	30
151-165	1584	1494	1639	1349	1078			1429	225		151-165	591	571	618	592	582			591	17
154-168	915	1230	1013	1871	1038			1213	385		154-168	412	570	494	559	472			501	65
157-171	1070	1071	764	1612	3943			1692	1295		157-171	594	572	533	640	550			578	42
160-174	784	1048	904	921	924			916	94		160-174	540	573	599	637	670			604	51
163-177	614	686	778	658	755			698	68		163-177	535	498	567	548	536			537	25
166-180	644	759	1967	5653	2811			2367	2044		166-180	482	503	562	549	533			526	33
169-183	1051	978	890	928	893			948	68		169-183	544	521	593	615	578			570	38
172-186	931	936	911	1061	916			951	62		172-186	523	505	587	515	549			536	33
175-189	2530	1231	1624	1330	1335			1610	535		175-189	613	609	572	616	592			600	18
178-192	1211	1132	965	804	1012			1025	157		178-192	541	554	591	662	585			587	47
TT	3152	2764	2246					2721	454		TT	23552	20403	22054					22003	1575
Rables	561	460	527	698	651			579	96		Rables	623	1491	724	2542	736			1223	815
Med	523	479	554	496	539			518	31		Med	659	549	625	577	625			607	44

HCO								Ave	SD
1-15	350	316	333	387	362			350	27
4-18	371	649	510	624	638			558	119
7-21	1167	494	831	872	602			793	261
10-24	1276	825	1050	1176	1128			1091	170
13-27	517	460	489	449	471			477	27
16-30	372	427	399	436	419			411	26
19-33	373	455	414	389	402			407	31
22-36	424	351	387	403	381			389	27
25-39	543	409	476	436	517			476	55
28-42	403	236	319	365	352			335	63
31-45	809	232	521	472	553			517	206
34-48	1372	1808	1090	1329	1315			1383	262
37-50	1087	301	694	847	749			736	286
40-54	344	386	365	372	346			363	18
43-57	539	418	478	438	492			473	47
46-60	554	621	587	576	563			580	26
49-63	1208	1307	1257	1341	1272			1277	50
52-66	686	393	540	561	571			550	105
55-69	375	403	469	416	399			412	35
58-72	492	461	469	498	473			479	16
TT	1590	1491	1436	1462				1495	67
Rabies	1244	460	2055	395	455	945		926	648
Med	272	301	346	296	279	326		303	28

Appendix C3

Proliferative response by HLA DR1 or DR4 subjects

Tritiated thymidine incorporation values (CPM) for healthy individuals and patients with the HLA DR1 or HLA DR4 haplotype. CPM values are initially given for the proliferative response to the peptide pools 1 - 14, tetanus toxoid peptide (TT) and HSP65 peptide (Hsp). Following this data are the CPM values for the proliferative response to individual peptides taken from pools 1 and 8 (amino acid range 1 - 72) and pools 3 and 10 (amino acid range 121 - 192). Background proliferation levels are given as medium (med).

DC*										Ave	SD
1	413	412	767	492	444	454	468	529		497	116
2	523	455	559	573	470	406	431	454		484	61
3	479	389	486	564	444	453	472	509		474	51
4	552	457	453	480	468	479	465	424		472	37
5	585	524	452	498	510	486	458	491		501	42
6	392	475	517	584	424	520	629	468		501	79
7	403	438	502	424	495	495	433	403		449	42
8	480	529	757	575	564	417	405	719		556	129
9	466	499	501	477	428	441	496	457		471	28
10	422	487	457	514	450	485	531	529		484	39
11	440	444	433	512	465	551	436	538		477	49
12	430	410	538	552	428	427	494	986		533	191
13	501	569	455	485	418	531	736	469		521	99
14	446	448	483	443	417	559	486	504		473	45
ttpep	544	490	565	496	441	411	547	543		504	56
HSp	549	528	527	483	478	572	453	448		505	46
TT	11979	11134	12871	11103	12297	7661	9215	9584		10731	1770
Rabies	769	663	392	633	574	511	537	705		598	120
Med	428	552	522	475	481	532	490	454		492	41

GG										Ave	SD
1	2141	658	1214	3088	1663	300	804	936	1679	669	1315	842
2	540	529	737	542	591	422	526	435	413	601	534	98
3	751	412	528	723	391	352	458	474	752	931	577	196
4	388	431	430	442	588	436	414	532	756	486	490	111
5	4567	1129	7892	658	557	645	692	670	767	604	1818	2459
6	536	740	773	769	668	991	640	662	434	531	674	157
7	634	553	541	517	502	608	423	535	444	562	532	65
8	1022	1058	1010	778	813	796	748	857	1064	741	889	134
9	411	569	472	383	499	544	486	484	432	729	501	98
10	384	464	552	548	352	398	536	485	491	464	467	70
11	890	868	804	518	555	518	467	478	823	778	670	176
12	452	1122	1812	926	1283	584	448	3531	4269	780	1521	1332
13	555	578	660	430	793	378	385	468	660	605	551	135
14	502	441	405	398	373	322	346	400	482	499	417	63
ttpep	572	426	552	376	510	500	447	361	556	526	483	76
HSP	440	642	449	494	381	518	534	480	516	428	488	72
TT	511	986	1991	1188	522	378					929	607
Rabies	617	536	527	515	454	364					502	85
Med	486	343	438	477	496	382	453	398	368	468	431	54

CR										Ave	SD
1	442	372	324	532	365					407	82
2	427	398	308	327	310					354	55
3	480	372	301	432	399					397	67
4	418	331	361	390	328					365	39
5	583	368	362	324	387					405	102
6	319	314	358	389	476					372	66
7	367	335	421	370	373					373	31
8	380	352	412	308	322					355	42
9	335	487	336	316	314					358	73
10	416	308	357	338	341					352	40
11	458	414	437	416	425					430	18
12	414	373	421	384	366					392	24
13	308	381	384	308	342					345	37
14	302	338	368	319	361					338	28
ttpep	381	370	362	374	330					364	20
HSP	360	443	417	453	385					411	39
TT	6355	6071	5977	6641	6261					6261	6237
Rabies	381	379	346	422	359					377	376
Med	492	312	395	312	369					376	74

DJ										Ave	SD
1	4956	4379	3882	9163	2421	2131	2113	4023		4134	2303
2	1532	1354	1356	1113	940	859	781	961		1112	273
3	966	1775	1423	1062	1174	868	935	1258		1183	301
4	1543	1677	1838	1599	2011	1677	1696	2325		1796	259
5	1667	1883	2285	1856	1159	994	1094	1424		1545	454
6	987	954	1022	1167	899	1008	1099	1114		1031	89
7	995	991	1250	994	1222	1124	1513	1240		1166	180
8	1036	1403	1726	3387	1369	1890	1351	1455		1702	727
9	2068	1328	992	1214	1293	1158	1179	1800		1379	364
10	2153	2373	967	1502	1821	1456	2179	1620		1759	467
11	1014	820	1922	1736	1654	1594	1625	2298		1583	472
12	2505	1555	1421	1513	1082	1629	1334	2032		1634	444
13	1763	1418	854	2618	1316	1305	1777	1063		1514	545
14	1366	990	858	1140	1776	1104	989	1684		1238	338
ttpep	2112	869	870	790	828	813	924	1007		1027	444
HSP	1243	1167	974	845	1060	958	933	973		1019	131
TT	22793	21026	23078	21775	21021	20247	18936	22212		21386	1375
Rabies	1492	908	926	1210	809	1449	1691	735		1153	359
Med	924	896	887	957	746	882	820	880		874	65

BC										Ave	SD		JK											Ave	SD
1	821	892	964	1107	974	1834				1099	373		1	1216	1063	1934	1008	269	250						957	634
2	1584	1007	1302	1240	859	925				1153	274		2	278	275	197	300	251	249						258	36
3	2212	1057	1123	1062	843	869				1194	511		3	209	240	193	198	257	234						222	26
4	953	785	734	852	1003	1501				971	278		4	198	195	206	265	201	270						222	35
5	1505	949	943	864	874	808				990	258		5	1228	399	1544	483	364	479						750	505
6	1614	1123	1273	1436	1504	963				1319	246		6	273	276	362	165	287	266						272	63
7	2422	1751	992	1059	1984	938				1524	618		7	173	225	247	306	212	318						247	56
8	2815	2587	1146	1006	853	805				1535	914		8	581	435	299	298	317	248						363	124
9	1949	967	1093	1452	856	961				1213	416		9	257	665	260	208	277	214						314	174
10	2679	859	1467	1142	3798	2905				2142	1159		10	242	264	246	268	242	297						260	21
11	987	942	881	1159	868	1012				975	107		11	259	268	238	239	240	189						239	27
12	1071	1405	1149	1375	874	1000				1146	210		12	199	177	188	274	182	205						204	36
13	1075	1037	1145	840	935	996				1005	107		13	188	269	221	262	260	213						236	33
14	980	914	826	879	994	1339				989	183		14	187	265	194	245	272	194						226	39
ttpep	988	937	883	1180	784	1171				990	158		ttpep	225	243	195	243	190	270						228	31
HSP	934	1029	1216	1176	908	875				1023	144		HSP	216	297	402	235	297	244						282	68
TT	7829	7272	7418							7507	7345		TT	5844	5546	6125	5982	6035	6249						5963	246
Rabies	891	1067	934	952	1035	911				976	980		Rabies	778	498	830	947	652	698	711					731	142
Med	804	913	995	1159	896	869				939	124		Med	187	209	224	288	246	289						240	42
GBA										Ave	SD														
1	157	180	193	202	168					180	18														
2	246	237	359	187	218					249	65														
3	207	142	191	192	174					181	25														
4	215	199	181	152	180					185	24														
5	420	426	341	450	395					406	41														
6	164	272	247	319	254					251	56														
7	200	209	185	278	194					213	37														
8	179	188	164	162	163					171	12														
9	187	161	173	195	186					180	13														
10	148	152	148	152	170					154	9														
11	167	197	193	188	174					184	13														
12	158	180	160	214	169					176	23														
13	254	177	198	185	224					208	31														
14	156	132	154	164	196					160	23														
ttpep	367	209	217	192	228					243	71														
HSP	226	167	195	225	214					205	25														
TT	4327	4812	4286	4878						4576	4659														
Rabies	374	571	454	545	531	457				495	512														
Med	196	169	172	175	153					173	15														

KD								Ave	SD
1	412	267	231	351	283			309	72
2	263	285	258	226	267			260	21
3	385	404	190	250	260			298	92
4	267	225	285	281	272			266	24
5	421	251	188	204	233			259	94
6	239	179	187	129	206			188	40
7	262	269	264	208	225			245	27
8	410	297	289	250	298			309	60
9	334	306	223	229	251			269	49
10	304	178	246	164	216			222	56
11	358	361	235	182	241			275	80
12	289	300	378	400	386			351	52
13	175	185	265	223	192			208	36
14	203	230	252	239	217			228	19
ttpep	182	164	185	229	207			194	25
HSP	172	150	150	206	182			172	23
TT	7684	7452	7924					7687	236
Rables	254	215	194	201	187			210	27
Med	174	106	163	207	194			169	39

LJ								Ave	SD
1	1450	1259	2008	1924	1943	2608		1865	474
2	1451	655	764	895	1020	880		944	278
3	6462	646	571	1466	5668	605		2963	2867
4	3708	723	573	1154	4720	697		2175	1907
5	3082	773	858	677	1748	2105		1428	1019
6	1681	836	733	602	3270	1720		1474	1005
7	671	714	760	629	603	879		676	64
8	898	814	771	808	691	795		796	75
9	777	932	836	628	984	981		832	139
10	893	755	677	1939	687	899		975	482
11	915	869	880	761	654	975		816	108
12	664	639	832	1136	8683	4653		2391	3523
13	701	3488	742	929	1965	1755		1565	1192
14	654	593	750	935	858	902		758	141
ttpep	714	715	982	1055	878	911		869	154
HSP	869	723	784	988	901	959		853	103
TT	7471	7861	8026					7786	285
Rables	812	844	1256	1067	982			992	180
Med	951	891	855	874	619	835		838	128

SH								Ave	SD
1	717	465	622	471	554			566	107
2	569	661	672	636	536			615	59
3	457	426	456	390	451			436	29
4	613	380	433	385	442			451	95
5	409	407	715	427	425			477	134
6	490	529	440	464	449			474	36
7	495	446	467	420	349			435	56
8	402	434	466	511	425			448	42
9	648	682	663	542	510			609	78
10	484	497	410	490	407			458	45
11	497	654	426	431	574			517	98
12	460	524	431	476	418			462	42
13	415	456	391	481	498			448	45
14	555	550	470	654	523			550	67
ttpep	486	500	615	506	426			506	68
HSP	477	534	530	500	448			498	36
TT	8472	7935	8215					8207	269
Rables	463	958	473	776	987			731	254
Med	433	330	462	499	474			440	66

KA								Ave	SD
1	448	283	461	530	198			384	138
2	230	206	291	387	322			287	72
3	262	323	340	282	329			307	33
4	250	235	332	290	411			304	71
5	396	317	622	557	498			478	122
6	301	264	266	318	426			315	66
7	201	174	214	205	218			202	17
8	248	155	246	243	213			221	40
9	221	264	248	246	273			250	20
10	385	440	295	447	385			390	61
11	327	297	487	376	290			355	81
12	200	278	207	321	236			248	51
13	316	270	324	247	342			300	40
14	165	228	197	237	177			201	31
ttpep	174	240	183	324	414			267	102
HSP	168	177	178	171	231			185	26
TT	8103	7676	7873					7884	214
Rables	280	286	367	347	291			314	40
Med	202	299	231	188	193			223	46

CM								Ave	SD		PBA								Ave	SD
1	309	306	362	403	227			322	66		1	633	683	591	741	678			665	56
2	354	330	318	354	284			328	29		2	1432	900	1209	1327	1052			1184	213
3	296	299	276	290	304			293	11		3	615	536	540	594	607			578	38
4	383	286	400	335	213			323	76		4	705	1112	1041	844	1028			946	167
5	336	394	355	565	406			411	91		5	612	573	687	596	618			617	43
6	451	380	296	329	356			362	58		6	1438	1218	1269	1179	793			1179	237
7	338	342	222	264	218			277	60		7	1096	1627	1499	1314	1226			1352	212
8	266	454	405	432	414			394	74		8	688	677	536	552	651			621	72
9	340	393	374	228	348			337	65		9	1591	1180	1271	1110	1304			1291	184
10	319	218	236	242	217			246	42		10	815	886	753	851	863			834	52
11	351	349	326	304	275			321	32		11	901	774	978	898	900			890	73
12	235	215	182	204	175			202	24		12	661	622	558	630	694			633	50
13	211	309	216	335	229			260	57		13	758	880	1028	826	966			891	108
14	264	295	324	233	317			287	38		14	935	1033	926	987	981			972	44
ttpep	255	318	329	348	253			300	44		ttpep	660	752	769	833	818			767	68
HSP	278	254	223	303	283			268	31		HSP	862	616	873	745	832			786	107
TT	13412	13837	13605					13618	213		TT	2857	2936	3009					2934	76
Rables	835	930	1016	789	806			875	96		Rables	1936	793	2029	942	1334	843		1313	553
Med	341	311	260	291	243			289	39		Med	656	588	606	610	698			632	45

SH							Ave	SD		CR							Ave	SD
1-15	430	532	627	654	496		548	93		1-15	376	377	412	365	395		385	19
4-18	379	512	461	389	424		433	55		4-18	369	348	359	421	396		379	30
7-21	634	505	827	542	576		617	127		7-21	449	360	362	433	416		404	41
10-24	412	444	425	446	436		433	14		10-24	400	382	443	458	422		421	31
13-27	464	427	468	428	437		445	20		13-27	363	398	400	391	374		385	16
16-30	432	450	451	406	442		436	19		16-30	354	357	401	369	400		376	23
19-33	441	424	483	520	474		468	38		19-33	432	384	351	411	406		397	31
22-36	861	476	469	510	467		557	171		22-36	445	382	422	717	497		493	132
25-39	784	647	1252	684	841		842	242		25-39	411	390	401	409	398		402	9
28-42	416	429	414	406	426		418	9		28-42	388	422	383	369	403		393	20
31-45	475	415	472	448	439		450	25		31-45	393	418	353	382	381		385	23
34-48	395	399	408	451	430		417	24		34-48	350	405	329	452	377		383	48
37-50	397	458	446	629	438		474	90		37-50	344	342	397	340	371		359	25
40-54	540	431	460	468	484		477	40		40-54	777	453	478	374	446		506	157
43-57	505	494	570	560	527		531	33		43-57	366	386	418	404	410		397	21
46-60	446	430	497	519	463		471	37		46-60	327	418	468	364	388		393	54
49-63	451	426	426	433	441		435	11		49-63	419	393	378	343	365		380	29
52-66	571	527	618	504	538		552	44		52-66	415	492	460	396	432		439	38
55-69	523	474	606	582	528		543	52		55-69	398	344	350	369	382		369	22
58-72	416	434	482	441	422		439	26		58-72	350	363	381	365	392		370	16
121-135	461	512	474	464	484		479	21		121-135	389	395	321	316	362		357	37
124-138	574	638	529	670	641		610	57		124-138	428	440	323	386	342		384	51
127-141	470	451	524	605	494		509	60		127-141	359	337	400	361	381		368	24
130-144	465	434	408	390	444		428	30		130-144	437	348	372	414	333		381	44
133-147	540	437	448	551	473		490	53		133-147	307	349	305	420	370		350	48
136-150	656	608	700	484	537		597	87		136-150	399	359	406	416	385		393	22
139-153	530	418	412	416	467		449	51		139-153	350	411	330	340	374		361	32
142-156	402	457	450	469	452		446	26		142-156	608	383	423	374	394		436	98
145-159	503	513	468	401	464		470	44		145-159	378	438	325	386	404		386	41
148-162	427	504	456	434	487		462	33		148-162	431	415	373	370	358		389	32
151-165	502	410	492	471	451		465	37		151-165	405	374	338	400	396		383	28
154-168	517	432	457	455	476		467	32		154-168	417	382	329	360	362		370	32
157-171	540	686	680	774	594		655	90		157-171	335	328	349	363	374		350	19
160-174	524	721	519	662	563		598	90		160-174	356	322	397	345	362		356	27
163-177	439	470	594	592	532		525	70		163-177	446	321	329	374	343		363	51
166-180	557	446	464	422	479		474	51		166-180	342	395	391	409	375		382	26
169-183	477	435	408	440	468		446	28		169-183	433	336	313	380	363		365	46
172-186	439	465	452	498	464		464	22		172-186	509	369	475	377	407		427	62
175-189	483	436	520	446	472		471	33		175-189	396	338	350	423	376		377	34
178-192	441	477	423	482	452		455	25		178-192	400	340	416	464	384		401	45
TT	8472	7935	8215				8207	269		TT	6355	6071	5977	6641	6261		4232	6134
Rabies	463	958	473	776	987		731	254		Rabies	381	379	346	422	359		338	368
Med	433	330	462	499	474		440	66		Med	492	312	395	312	369		376	74

Appendix C4

Proliferative response by HLA DQ2 or DQ6 healthy individuals

Tritiated thymidine incorporation values (CPM) for healthy individuals with the HLA DQ2 haplotype. CPM values are initially given for the proliferative response to the peptide pools 1 - 14, tetanus toxoid peptide (TT) and HSP65 peptide (Hsp). Following this data are the CPM values for the proliferative response to individual peptides taken from pools 1 and 8 (amino acid range 1 - 72) and pools 3 and 10 (amino acid range 121 - 192) . Background proliferation levels are given as medium (med).

MD															Ave	SD
1	951	923	839	484	577	1346	698	695	476	561	787	1311	804	391	774	290
2	367	359	393	339	312	411	417	300	338	306	370	447	313	368	360	45
3	765	484	360	387	626	317	432	409	582	706	444	689	508	461	512	139
4	611	369	416	384	377	401	411	471	492	336	530	389	316	441	425	79
5	911	687	388	500	632	695	574	848	875	660	773	734	686	461	673	155
6	366	362	350	371	355	319	350	414	382	489	382	358	325	360	370	41
7	369	811	466	1124	458	332	450	376	753	308	379	348	473	383	502	233
8	823	848	548	712	856	864	1054	776	869	913	581	663	792	444	767	162
9	338	387	330	470	327	429	398	341	402	407	399	547	331	405	394	62
10	683	727	544	742	860	782	787	628	792	569	1310	637	755	400	730	207
11	455	424	652	450	379	548	368	317	782	684	507	482	420	432	493	132
12	322	508	450	357	327	422	316	335	321	338	432	319	312	386	368	62
13	460	508	442	357	416	414	369	305	338	325	384	533	404	372	402	66
14	466	872	365	467	433	361	360	457	434	434	408	449	442	353	450	128
ttpep	400	493	1404	422	349	585	418	446	436	620	496	514	524	301	529	266
HSP	394	543	359	442	334	340	329	490	436	386	276	356	390	376	389	70
TT	60902	69294	64328	65439											64991	3458
Rabies	1668	1611	842	793	1538										1291	476
Med	488	395	602	474	487	309	660	630	712	690	375	344	522	373	504	136

PW*															Ave	SD
1	4853	5827	6136	6455	6067	7403	2593	2519	2566	1999					4642	2017
2	1393	1524	831	1118	1121	822	792	1323	1458	1183					1156	272
3	1634	3006	2361	2982	2976	1657	2596	986	2380	2038					2262	680
4	1362	1747	1018	1488	1702	940	1124	1138	2094	1384					1400	366
5	2275	3522	1924	2314	2265	2002	2260	2439	3015	2104					2412	491
6	953	1402	1349	1782	1512	1223	984	1820	1806	1226					1406	323
7	1077	1898	1455	1794	1869	1908	1411	1970	1585	1456					1642	291
8	4409	2666	2861	4009	2965	3589	2843	3793	5247	3394					3578	817
9	947	1266	995	880	848	982	894	999	1166	1090					1007	132
10	3199	3777	3408	4282	3762	2099	3012	3306	4978	2649					3447	815
11	1989	2338	1234	2314	2120	1586	1280	1645	1596	1461					1756	407
12	1499	2507	1353	2258	2377	1936	1579	1890	1657	2596					1965	446
13	1905	1355	932	1382	1168	1148	1565	1352	1069	1347					1322	274
14	963	914	820	1053	1102	851	1172	1062	1374	1398					1071	199
ttpep	2015	1576	1057	1742	1474	1164	1077	1928	984	1268					1428	376
HSP	2120	2678	1563	2699	2040	1764	1881	2036	1974	2183					2094	361
TT	19067	19684	20678												19810	813
Rabies	970	1029	944	1354	964										1052	190
Med	898	1033	852	1049	950	891	818	1133	985	1119					973	110

CC*											Ave	SD
1	593	693	293	558	448	295	467	381			466	143
2	234	221	227	287	294	291	514	402			309	101
3	464	305	284	366	311	250	437	343			345	74
4	364	295	279	268	315	264	625	445			357	124
5	399	225	212	260	277	318	274	296			283	58
6	316	304	310	251	228	324	386	355			309	51
7	327	283	305	278	243	436	357	396			328	65
8	375	687	431	362	342	788	610	699			537	179
9	313	332	273	293	351	397	337	367			333	40
10	695	785	740	580	622	448	442	445			595	139
11	244	303	274	289	265	372	428	400			322	69
12	298	294	296	310	303	268	291	280			292	13
13	220	254	237	228	246	254	288	271			250	22
14	246	352	299	267	273	318	326	322			300	36
ttpep	357	492	425	480	461	310	372	341			405	69
HSP	561	292	427	376	625	332	312	299			403	126
TT	6875	5983	6429								6429	446
Rabies	1233	1811	700	515	951						1042	583
Med	256	261	308	289	328	286	313	306			293	25

SH											Ave	SD
1	379	463	356	337	369						381	49
2	361	335	376	385	343						360	21
3	334	319	431	366	347						359	44
4	474	368	391	378	360						394	46
5	373	404	315	361	343						359	33
6	412	366	426	344	395						389	33
7	361	359	349	365	391						365	16
8	447	348	371	351	422						388	44
9	432	438	373	365	401						402	33
10	420	348	382	364	410						385	30
11	363	416	371	352	394						379	26
12	341	443	427	391	368						394	42
13	402	408	406	386	368						394	17
14	349	335	443	365	395						377	43
ttpep	365	325	398	396	373						371	30
HSP												
TT	12558	12764	13892								13072	718
Rabies	572	644	551	482	524						555	67
Med	386	444	347	406	371						391	37

ML															Ave	SD
1	1189	1965	1346	2179	1259	1690	1270	2108	2709	2378	2407	3110	1967	628	1872	680
2	841	823	422	495	606	466	597	794	765	726	760	705	650	464	651	144
3	2187	1769	1199	1018	596	1068	793	808	1166	987	1438	2153	1265	522	1212	519
4	463	656	464	492	504	454	446	561	760	902	807	747	596	573	602	149
5	1397	1130	1051	1218	1067	1249	723	1439	1432	1394	1391	1501	1249	526	1198	284
6	657	596	544	702	494	508	717	897	836	677	791	839	696	577	681	128
7	509	586	483	504	434	640	549	578	704	507	865	946	609	219	581	178
8	1232	1215	1140	1505	1035	1469	1033	1413	2479	1790	1959	3220	1624	1657	1626	605
9	954	590	596	722	421	531	522	990	921	579	594	799	768	410	671	190
10	1241	1450	972	1184	850	1518	1160	1318	1730	1002	2398	1686	1376	1424	1379	390
11	538	733	598	609	588	695	542	686	831	579	923	815	703	570	672	119
12	487	1045	571	1100	651	840	528	624	1578	1124	747	893	932	564	835	305
13	405	573	491	666	423	491	455	506	810	475	668	735	558	531	556	121
14	456	806	611	614	670	622	712	602	645	456	621	738	704	512	626	101
ttpep	1483	1809	2082	3650	2212	1086	1179	3034	2024	2984	1280	4154	2248	1002	2159	980
HSP	504	469	576	455	542	469	480	421	514	457	492	533	418	494	487	45
TT	52180	59556	50014												53916	5003
Rabies	405	712	1456	2158	1064										1159	786
Med	538	463	481	428	458	490	428	518	460	450	477	566	455	492	479	40
CL																
1	3275	1860	3249	4786	4069	3117	2064	4518							3367	1061
2	865	786	774	888	754	604	768	742							773	86
3	1298	1307	1411	966	1130	1047	1159	1078							1174	151
4	3923	863	1119	3129	1099	677	649	759							1527	1264
5	3769	7091	4246	2149	1576	3193	4627	1899							3569	1810
6	1092	963	1051	963	1542	713	971	719							1002	259
7	1253	893	766	1107	743	645	1513	993							989	292
8	1827	7190	3916	8756	3334	1459	7557	6280							5040	2768
9	836	851	1223	610	703	711	570	651							769	208
10	3397	1076	2299	1003	2059	1375	830	1356							1674	863
11	1508	1505	1504	1123	938	1447	653	846							1190	346
12	2302	2493	4446	3424	6769	2392	747	2292							3108	1816
13	1124	631	1381	922	1226	876	538	995							962	286
14	1383	749	671	890	681	636	643	750							800	249
ttpep	1154	944	1070	884	839	1022	743	1434							1011	215
HSP	1388	957	1327	1675	1754	892	1155	1476							1328	313
TT	47456	48221	45106												46928	1623
Rabies	1357	6148	2074	5187	1358										3225	2334
Med	598	495	591	713	612	684	653	569							614	69

AG										Ave	SD
1	1922	2406	1824	2012	1969	5182				2552	1304
2	352	390	333	339	363	467				374	50
3	878	838	738	1158	1232	1098				990	199
4	540	395	482	605	467	404				482	80
5	1665	2304	1480	2315	2367	1696				1971	399
6	479	328	317	376	318	393				368	63
7	498	369	309	335	393	274				363	78
8	2770	2124	3150	2654	2133	2571				2567	393
9	452	486	396	466	312	446				426	64
10	1305	964	1231	3454	1026	1045				1504	964
11	459	550	443	423	416	545				473	60
12	981	1013	918	994	1668	1060				1106	279
13	432	542	428	465	472	504				474	44
14	454	439	473	500	408	520				466	41
ttpep	453	342	459	672	686	435				508	139
HSP	989	838	684	472	435	352				628	251
TT	47456	48221	45106							46928	1623
Rabies	1482	413	472	1665	575					1997	554
Med	406	420	435	464	414	365				417	33
GB											
1	677	938	821	422	477	443				630	217
2	329	517	638	434	520	497				489	103
3	739	607	862	635	815	663				720	103
4	436	523	621	516	503	456				509	65
5	12016	10495	6414	1584	2530	1650				5782	4621
6	816	864	1102	993	776	858				902	122
7	597	489	610	642	595	483				569	67
8	1188	1156	869	677	714	830				906	218
9	498	592	482	536	451	515				512	49
10	513	836	840	660	687	712				708	122
11	474	464	504	520	620	484				511	57
12	2636	2031	1027	1690	967	2243				1766	670
13	448	498	882	401	944	671				641	231
14	449	518	484	452	527	478				485	33
ttpep	536	644	563	697	540	727				618	83
HSP	506	407	459	536	602	632				524	85
TT	5776	8099	6441	5810	7635					6752	1089
Rabies	640	608	494	476	608	580				568	82
Med	450	416	354	397	479	422				420	43

AG									Ave	SD
1-15	411	458	393	438	371	394			411	32
4-18	388	403	444	412	395	408			408	20
7-21	470	427	392	370	370	406			406	38
10-24	435	399	376	372	386	414			397	24
13-27	414	427	460	379	412	419			418	26
16-30	334	331	398	366	404	407			373	35
19-33	434	477	399	397	428	427			427	29
22-36	448	450	382	415	425	364			414	35
25-39	465	679	424	398	539	501			501	101
28-42	419	444	376	389	376	401			401	27
31-45	813	1008	823	842	1583	1014			1014	293
34-48	404	366	484	358	422	407			407	45
37-50	361	367	417	466	397	402			402	38
40-54	440	449	372	423	379	393			409	33
43-57	405	420	371	435	468	400			416	33
46-60	380	399	393	456	371	380			396	31
49-63	472	534	567	1013	392	596			596	217
52-66	399	381	374	449	392	379			396	28
55-69	392	442	391	447	384	411			411	27
58-72	452	431	412	463	413	414			431	22
121-135	432	407	410	429	440	424			424	13
124-138	363	380	429	374	409	371			388	26
127-141	433	467	401	382	370	431			414	36
130-144	481	367	342	412	409	422			406	48
133-147	361	406	363	404	433	453			403	37
136-150	389	402	497	394	424	401			418	41
139-153	422	409	459	368	381	368			401	36
142-156	391	362	427	378	423	396			396	25
145-159	329	500	572	406	583	478			478	98
148-162	471	451	373	457	363	383			416	48
151-165	1318	1891	1220	1110	828	1273			1273	350
154-168	373	461	406	376	369	377			394	36
157-171	355	446	475	409	457	368			418	49
160-174	383	388	395	428	433	395			404	21
163-177	438	412	363	417	407	408			408	25
166-180	381	336	359	398	464	407			391	44
169-183	340	446	377	373	422	472			405	50
172-186	375	421	393	398	413	400			400	16
175-189	377	412	368	358	390	461			394	38
178-192	421	444	406	448	382	381			414	29
TT	47456	48221	45106						46928	1623
Rabies	1482	413	472	1665	575				1997	554
Med	406	420	435	464	414	365			417	33

ML											Ave	SD
1-15	529	429	454	491	460	510	476	611	512		497	53
4-18	488	496	508	482	494	530	448				492	25
7-21	530	525	546	500	486	525	480	493	535		513	24
10-24	504	511	470	527	498	485	518				502	20
13-27	505	669	521	519	497	491	497	482	484		518	58
16-30	488	535	434	482	509	459	590				500	51
19-33	517	469	476	496	502	476	528	483	423		486	31
22-36	412	504	516	462	517	522	458				484	41
25-39	508	483	519	497	474	453	449	468	456		479	25
28-42	613	543	499	464	549	497	483				521	51
31-45	528	447	537	489	507	461	523	519	495		501	31
34-48	485	484	513	484	487	523	456				490	22
37-50	460	521	513	522	508	474	538	514	486		504	25
40-54	432	456	506	517	501	463	522				485	35
43-57	497	503	536	510	499	531	454	497	534		507	26
46-60	500	443	461	498	507	523	506				491	28
49-63	509	499	527	496	535	464	466	450	455		489	32
52-66	451	485	460	468	500	452	476				470	18
55-69	465	453	468	539	523	440	510	646	465		501	64
58-72	550	480	538	451	441	512	516				498	42
121-135	1111	416	458	444	522	478	468	482			547	230
124-138	473	406	452	534	516	480	442	684			498	85
127-141	503	450	503	499	517	456	483	473			486	24
130-144	480	460	475	461	431	475	514	424			465	29
133-147	434	493	473	431	526	474	502	436			471	35
136-150	476	448	530	517	423	458	474	463			474	35
139-153	442	475	430	470	460	420	491	496			460	28
142-156	459	513	468	472	487	519	433	459			476	29
145-159	451	496	485	500	513	497	421	436			475	34
148-162	515	498	430	502	476	507	518	501			493	29
151-165	473	534	453	425	441	504	491	470			474	35
154-168	439	454	531	496	434	488	530	455			478	39
157-171	492	473	463	444	421	505	538	467			475	36
160-174	475	493	478	487	508	518	436	486			485	25
163-177	586	458	470	514	480	468	463	487			491	42
166-180	467	517	432	493	448	433	429	443			458	32
169-183	609	493	467	428	532	463	492	463			493	56
172-186	479	469	498	472	433	477	497	504			479	23
175-189	441	478	477	443	498	455	518	469			472	27
178-192	411	472	472	469	488	506	481	495			474	29
TT	37458	42075	39457								39663	2315
Rabies	11286	15012	618	2974	1078						6194	6544
Med	571	591	538	627	561	594	607	519			576	36

PW									Ave	SD
1-15	985	842	961	1002	911	914	946		937	54
4-18	942	974	968	1024	853	894			942	61
7-21	969	932	887	899	935	986	958		938	36
10-24	942	1007	871	971	920	855			928	58
13-27	1016	936	943	962	958	954	1011		969	32
16-30	830	960	858	986	1006	925			928	71
19-33	881	911	1028	878	923	999	970		941	59
22-36	873	998	864	945	952	943			929	51
25-39	937	943	888	944	957	996	919		941	33
28-42	1012	968	912	998	988	849			954	62
31-45	904	896	920	926	850	926	1000		917	45
34-48	903	885	940	1162	933	965			965	101
37-50	969	920	974	930	962	1067	904		961	54
40-54	910	835	942	946	1005	946			931	56
43-57	929	870	993	1103	935	980	902		959	76
46-60	912	993	944	875	929	984			940	44
49-63	851	933	3230	966	916	3121	913		1561	1104
52-66	913	897	1038	989	908	882			938	62
55-69	869	910	919	908	1002	970	954		933	45
58-72	915	972	1018	968	911	873			943	53
121-135	952	869	964	960	1056	996			966	61
124-138	984	954	1005	880	954	918			949	45
127-141	968	1137	950	945	993	955			991	73
130-144	997	907	957	1000	918	992			962	41
133-147	990	883	999	1022	962	886			957	59
136-150	846	962	900	1186	925	934			959	118
139-153	950	938	898	910	972	906			929	29
142-156	899	980	1106	944	991	853			962	87
145-159	1038	987	919	885	953	963			958	53
148-162	953	934	853	956	987	885			928	50
151-165	1155	902	903	966	962	901			965	98
154-168	986	991	921	995	1118	962			996	66
157-171	934	923	905	933	1036	879			935	54
160-174	906	918	944	933	846	986			922	46
163-177	933	1203	905	884	972	929			971	117
166-180	918	878	927	883	935	908			908	23
169-183	932	958	936	980	984	1113			984	67
172-186	953	932	953	951	867	916			929	34
175-189	961	980	1058	975	998	933			984	42
178-192	972	868	989	1009	869	898			934	63
TT	16487	15247	15836						15857	620
Rabies	638	751	594	578	524				617	85
Med	567	538	645	546	610	537			574	44