ANTIGEN PROCESSING PATHWAYS IN
TRANSPLANTATION

by

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A Thesis submitted to the University of London in partial fulfilment for the degree of Doctor of Philosophy with the Faculty of Medicine.

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SECTION A - ABSTRACT

Antigen presentation and its regulation has been studied, with particular emphasis on the control and function of the MHC class II molecule, and on how this may affect allograft rejection. CIITA is a specific controller of class II expression, and has recently been cloned in the human, using cell lines derived from MHC class II deficient patients. We have started to clone the gene for rat CIITA, using PCR techniques, and have isolated a 433 base pair fragment. The full length CIITA sequence would be used to manipulate class II expression in experimental models, using, initially, antisense oligonucleotides.

In the interim, we have gained experience with antisense technology by targeting the IL-2 molecule. This cytokine is integral to the interaction between an APC presenting peptide on its surface, and the T-cell. We have attenuated T-cell proliferation in in vitro assays, and show evidence that this is due to specific down-regulation of IL-2 production.

In examining the intermediate steps between CIITA and class II expression, we have focused on the invariant chain. The invariant chain defines the ability of the class II molecule to present exogenous peptide. Its distribution in normal rat organs and transplanted kidneys is described. The results are unexpected, and the possible significance of the invariant chain in influencing self and non-self peptide recognition is discussed.

Additionally, we report a new group of patients with MHC class II deficiency, from ethnic groups not previously seen to be affected, and immunohistology studies on fibroblast cell lines from these patients show a complete lack of invariant chain expression.

Finally, we have looked for the presence of indirect allorecognition in the clinical situation of graft rejection. We have recruited patients undergoing follow-up for chronic rejection, and performed an in vitro assay to detect a T-cell response indicating the presence of indirect recognition. For reasons that are discussed, the results are negative.
Acknowledgements

I would like to thank my supervisors, Professor John Fabre and Dr. Kenth Gustafsson, for their invaluable help and discussion during this project.

The members of the transplantation biology team should also all take collective responsibility for attempting to transform me into a scientist. Thanks particularly to Ged Murphy, Andy Preece and Karen Strahan for dispensing on me all they know about molecular biology, to Lorna Shewring for being my tissue culture guru, and to Greta Sawyer and Rebecca Schofield for helping out with the animal work. Much appreciation also to Sheng Yun for being an example of superhuman productivity in the lab, and to Rosie Dalchau for endless moral support and advice on half-timbered barn conversions, and also to Sally Cunningham and Gabi Slapak for attempting to dispel the myth that Medics can also do science. Adam Benham is also sorely missed, if only for his self-directed cries of ‘rubbish!’ on the football pitch.

Finally I would like to thank my family; my parents for successfully cloning themselves into me, and then accepting the consequences; Maryam and Safya for providing constant distraction whilst trying to write up, and my wife, Jane, for her support and trips to Sainsbury’s.

Funding for this project was kindly supplied by the National Kidney Research Fund.
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<th>Description</th>
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<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
</tr>
<tr>
<td>β₂m</td>
<td>β₂ microglobulin</td>
</tr>
<tr>
<td>BLS</td>
<td>Bare lymphocyte syndrome</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II transactivator</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II loading invariant chain peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte associated antigen</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytosine triphosphate</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxy ATP</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ddATP</td>
<td>dideoxy ATP</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTH</td>
<td>Delayed type hypersensitivity</td>
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<td>E.coli</td>
<td><em>Escherichia coli</em></td>
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<td>ELAM-1</td>
<td>Endothelial leucocyte adhesion molecule-1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>gram or gravitational acceleration</td>
</tr>
<tr>
<td>GTC</td>
<td>guanidinium thiocyanate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>ICAM-1,2</td>
<td>Intercellular adhesion molecule-1, 2</td>
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<tr>
<td>IDC</td>
<td>Interstitial dendritic cell</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>li</td>
<td>Invariant chain</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-2R</td>
<td>Interleukin-2 receptor</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLC</td>
<td>Mixed lymphocyte culture</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>O.D.</td>
<td>Optical density</td>
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<tr>
<td>Oligo</td>
<td>Oligodeoxynucleotide</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood leucocyte</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>RAM</td>
<td>Rabbit anti-mouse</td>
</tr>
<tr>
<td>rIL-2</td>
<td>Recombinant IL-2</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RPE</td>
<td>R-Phycoerithrin</td>
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<td>RSV</td>
<td>Respiratory syncitial virus</td>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Th1/Th2</td>
<td>T-helper 1 / T-helper 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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<tr>
<td>TTP</td>
<td>Thymine triphosphate</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular adhesion molecule-1</td>
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<tr>
<td>VLA-4</td>
<td>Very late activation antigen-4</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
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## ONE LETTER AND THREE LETTER CODE FOR AMINO ACIDS

### Neutral and hydrophobic:
- Alanine (Ala)\(^{A}\), Valine (Val)\(^{V}\), Leucine (Leu)\(^{L}\), Isoleucine (Ile)\(^{I}\), Proline (Pro)\(^{P}\), Tryptophan (Trp)\(^{W}\), Phenylalanine (Phe)\(^{F}\), Methionine (Met)\(^{M}\)

### Neutral and Polar:
- Glycine (Gly)\(^{G}\), Serine (Ser)\(^{S}\), Threonine (Thr)\(^{T}\), Tyrosine (Tyr)\(^{Y}\), Cysteine (Cys)\(^{C}\), Glutamine (Gln)\(^{Q}\), Asparagine (Asn)\(^{N}\)

### Basic:
- Lysine (Lys)\(^{K}\), Arginine (Arg)\(^{R}\), Histidine (His)\(^{H}\)

### Acidic:
- Aspartic Acid (Asp)\(^{D}\), Glutamic Acid (Glu)\(^{E}\)
**AMINO ACID TRANSLATION OF NUCLEOTIDE TRIPLET CODONS**

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<thead>
<tr>
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<td>GAA</td>
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<tr>
<td>UUC</td>
<td>UAC</td>
<td>GAG</td>
</tr>
<tr>
<td><strong>Leucine</strong></td>
<td><strong>Proline</strong></td>
<td><strong>Cysteine</strong></td>
</tr>
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<td>UUA</td>
<td>ACU</td>
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<tr>
<td>CUA</td>
<td>ACG</td>
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<tr>
<td>CUG</td>
<td>Serine</td>
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<td><strong>Valine</strong></td>
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<td>GCC</td>
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<tr>
<td><strong>Alanine</strong></td>
<td><strong>Asparagine</strong></td>
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<td>AAU</td>
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<td>UCA</td>
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<td>AAA</td>
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<td></td>
<td>Threonine</td>
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<td>Termination codon</td>
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Termination codon
SECTION B - LITERATURE REVIEW

B.1 ANTIGEN PRESENTATION

B.1.1 INTRODUCTION

Sixty years ago, an antigen was discovered by Gorer, on all the cells of a mouse, that was recognised by the immune system during rejection of a foreign tissue transplant. This work led to an explosion in the field of transplantation immunology, and Gorer’s antigen evolved into the major histocompatibility complex (MHC) of the mouse, later renamed H-2. During the 1960’s, another class of antigens was described, that turned out to be associated with the MHC. These, class II antigens, are principally restricted to certain cells of the immune system, namely lymphocytes and reticular cells.

Fragments of proteins, be they derived from breakdown of intrinsic cellular products, or from foreign material entering the body, are processed through specialised cells and presented on the cell surface as peptide in association with one of two types of this polymorphic molecule, the MHC molecule. This process enables the recognition of that peptide by a receptor molecule on T-cells, and the subsequent activation of the immune network of cells, in order for the appropriate response to be mounted to that antigen. The very complexity of the system inherently allows for both a specific and also a highly adaptive immune response, and although much of the detail has been painstakingly elucidated over the past few decades, as will be discussed there is much of the jigsaw yet to be put together, as
well as new pieces to discover. The system is not a linear one, and each fragment of new information often reveals more questions than it answers.

The first realisation that T-cells respond to proteins and show a completely distinct pattern of antigen reactivity from that shown by B-cell antibodies was in the 1960’s and 1970’s (Rosenthal et al. 1973). The most remarkable finding was that T-cell responses were limited by and restricted to allelic forms of a set of highly polymorphic glycoproteins encoded in the genetic region termed the major histocompatibility complex (MHC) (Katz et al. 1973). In the following decade further key findings were made, in particular that the MHC molecules were peptide binding proteins that presented a fragment of the original protein antigen bound tightly in their polymorphic region (Babbitt et al. 1985), and that the ‘dual specificity’ of T-cells for antigen and MHC molecules was made possible by a single receptor structure, the αβ TCR (Yague et al. 1985). The ensuing question was: why two distinct classes of MHC molecules, with different protein domains have evolved. The answer seems to be that each of the two classes of MHC molecules focuses on capture and presentation of peptides in distinct intracellular locations (Germain. 1986). Class I molecules appear committed to peptides present in the endoplasmic reticulum (ER), derived from proteins synthesised by the cell or entering the cytosol (Yewdell et al. 1988). To complement this, class II molecules seem concerned primarily with peptides reaching the endocytic pathway, most often from exogenous protein sources (Morrison et al. 1986).
B.1.2 THE MHC CLASS I PATHWAY OF ANTIGEN PRESENTATION

B.1.2.1 Class I structure

MHC class I molecules consist of a polymorphic type I integral membrane glycoprotein heavy chain of about 46 kDa, noncovalently associated with a 12kDa soluble subunit, β₂-microglobulin (β₂m) (Bjorkman et al. 1990). High resolution x-ray crystallographic structures have been derived for human and mouse class I molecules (Bjorkman et al. 1987; Fremont et al. 1992). The most striking aspect of these structures is that the α₁α₂ domain unit forms a single peptide binding site supported by a β-pleated sheet floor containing eight strands and bounded by two α-helices, one from α₁ and one from α₂. β₂m makes contact with both the immunoglobulin-like α₃ domain, and also the floor of the peptide binding region. There also exists a set of pockets, in some cases extending deep between the floor and helical walls of the binding region (Garret et al. 1989).

B1.2.2 The nature of peptide binding to the class I molecule

The most striking characteristic of the MHC class I molecules is their profound polymorphism. The determination of the sequences of peptides eluted from class I molecules, and of the crystal structure, has yielded a model for the way this polymorphism affects antigen presentation and recognition. Important general characteristics of self-peptides bound to class I were identified by Falk (Falk et al. 1991) who subjected pools of peptides to Edman degradation. Although unique sequences were not obtained from these heterogeneous mixes, particular amino acids gave very strong signals at specific positions. The predominant residues could
be found in comparable positions in most peptides, giving rise to the concept of ‘motif’ amino acids important in promoting binding to a particular allele of class I. The lack of strong signals beyond 9 amino acids in these pools of eluted material fit well with the 8 and 9 residue length of specific peptides found associated with class I molecules, suggesting that length is important for tight peptide association.

Allelic polymorphism also contributes to the shape and physico-chemical character of the pockets within the binding region, and notably almost all the side chains of the polymorphic amino acids in the binding domain are orientated inward toward the region containing peptide (Bjorkman et al.1987). This suggests that the major role of polymorphism is to regulate the binding of peptides, rather than to directly affect interaction with the T-cell receptor. In fact the α1α2 domain is crucial in selective peptide binding, and mutation in this region has been shown to abolish peptide recognition by T-cells (Moots et al.1991).

The nature of peptide visualised in HLA-B27 electron density maps has permitted detailed model building of a prototype bound peptide to class I (Madden et al.1991). The consensus peptide is nine residues long, and in an extended conformation with a central kink. A critical aspect of the peptide binding is the presence of conserved hydrogen bonds to the peptide’s NH₂ and COOH termini through conserved MHC residues. Such bonds, common to all peptides, suggest how otherwise polymorphic class I molecules can each act as effective peptide binding proteins for a wide diversity of peptide sequences.

Several groups have used related strategies to identify and characterise class I-associated peptides. For example more than 200 different peptides have been co-purified with HLA-A2 (Hunt et al.1992). More recently motifs of several other
class I bound peptides have been determined, and of those peptides whose source protein could be identified, all but one are from abundant cytoplasmic or nuclear proteins (Wei et al.1992).

B1.2.3 Peptide transport within the cell

The molecule responsible for transmitting peptides from the cytosol to the ER has been termed TAP, the transporter associated with antigen processing. This molecule was identified by studying class I deficient mutant cell lines (Spies et al.1990). TAP is a heterodimer, with both subunits (TAP1 and TAP2) being encoded in the MHC, and co-expression of both required for peptide transport (Neefjes et al.1993). The molecule is necessary for efficient peptide transport into the ER, with competition studies suggesting that there is only a single binding site for peptide (Heemels et al.1993). There are several alleles of both human TAP-encoding genes, but no evidence of this accounting for different substrate specificity. As would be expected, TAPs are able to transport a diverse set of peptides, including those that are longer by a few residues than those bound to class I (Momburg et al.1994). There is evidence that longer peptides may be trimmed in the ER (Roelse et al.1994). TAP molecules associate in the ER with class I molecules (Ortmann et al.1994), and this association can occur with the TAP1 subunit in the absence of TAP2 expression (Androlewicz et al.1994). Thus the TAP molecule enables the monitoring of the internal contents of intact cells, by transferring the information content from the cytosol to the ER, and may also play a part in limiting the repertoire of peptides transported, so participating in thymic T-cell repertoire selection (Momburg et al.1994).
B1.3 THE MHC CLASS II PATHWAY OF ANTIGEN PRESENTATION

B1.3.1 Structure of the class II molecule

MHC class II molecules are type I heterodimeric integral membrane proteins. Each dimer consists of one α and one β chain in noncovalent association. As with class I, the intron/exon organisation of genes encoding these chains corresponds to functional domains of the protein molecule, with the second exon containing coding information for the bulk of the positions at which extensive intraspecies polymorphism exists (Choi et al. 1983).

Six years after the publication of the three-dimensional structure of a MHC class I molecule, the human MHC class II structure was described by the same laboratories (Brown et al. 1993). In peptide-MHC interactions, the fundamental difference shown between class I and class II molecules, is in the peptide binding pocket. The class I pocket is blocked at either end and imposes severe size restrictions on the sizes of peptides it can accommodate, with longer peptides bulging out in the middle (Bjorkman et al. 1990), whereas the class II binding groove allows peptide to protrude from it. Consequently, longer (average 15-18 residues) peptides can bind, with no bulges needed. In addition, promiscuous peptides, capable of binding to many different human class II alleles, have been identified, with no obvious parallel in class I products (Sinigaglia et al. 1988). The other major surprise revealed was that the DR1 heterodimer occurs as a dimer of dimers, with an orientation that would allow interaction with two TCR complexes. This may lead to an increased affinity for the CD4 receptor and to the cross-linking of T-cell receptors to initiate cytoplasmic signalling pathways.
B1.3.2 Peptide and MHC class II transport

The class II α and β subunits associate rapidly in the ER, in the presence of the invariant chain (Ii) to form a trimer. The role of Ii in class II transport and function is fully reviewed in section B.3. The class II molecule/Ii complex leaves the ER, and reaches the endosomes via the golgi apparatus, whereupon Ii is cleaved leaving the class II molecule free to bind with processed exogenous peptide.

The delivery of processed antigen generated in lysosomes to available class II binding sites in late endosomes may be the responsibility of a pair of molecules termed p72/74 that are members of the hsp70 family (Vanbuskirk et al.1989). These have been shown to possess peptide binding capacity, and it is suggested that they might scavenge peptide or partially degraded proteins, preventing degradation in the proteolytically active environment of these endosomes, and shuttle the peptides to class II accumulated in earlier compartments. There is, interestingly, evidence for structural homology between the peptide binding regions of hsp70 molecules and MHC molecules (Rippmann et al.1991). A defect in this type of transport mechanism could give rise to the defective exogenous antigen presentation seen in certain mutant cells (Riberdy et al.1992), and superficially appears homologous to the TAP mutants with defective class I antigen presentation (Henderson et al.1992). The mutations in both cases overlap in the genome (Spies et al.1990; Mellins et al.1991), consistent with a cluster of peptide transport genes in this region of the MHC.
B1.3.3 Characteristics of peptide binding to the MHC class II molecule

Extensive intraspecies polymorphism is also a hallmark of MHC class II molecules, which plays a major role in determining which peptides show long-lived binding to class II molecules (Buus et al. 1987). Single residue changes in the floor of the binding site or in the helices can decrease binding of certain peptides by several orders of magnitude (Brett et al. 1989).

Peptides eluted from purified class II molecules demonstrate that each MHC allele gives a characteristic profile of eluted peptides, which are longer and more heterogeneous in size than those associated with class I molecules, ranging in length from 12 to more than 20 residues (Rudensky et al. 1991). This reflects the capture of nested sets of peptides from a single protein, and also the open ended structure of the class II binding groove. Motifs for binding to particular class II alleles have been identified by studying overlapping sequences of eluted peptides. These generally involve 2-3 residues from among a core region of 7-9 residues, and they occupy polymorphic pockets in the binding groove as with class I (Rudensky et al. 1992). The motifs consist of several peptide positions where amino acids with similar side chains occur with increased frequency (anchor positions). There also appear to be inhibitory residues, found more frequently at anchor positions (Hammer et al. 1994), implying that the binding of a given peptide to a certain class II allele is the result of both attracting and repelling forces. The predominant identifiable peptides bound to class II molecules are derived from proteins with ready access to the endocytic pathway, and more specifically, the majority appear to source from MHC-related molecules (Chicz et al. 1992a). Very few of the less abundant peptides associated with class II have been identified. Several
promiscuous peptides, capable of binding to many different class II alleles, have been identified (Sinigaglia et al. 1988). Promiscuous peptides should contain either overlapping class II binding motifs, or they should use anchors that are conserved among DR ligands, and should lack allele-specific contact sites that could prevent binding to other class II molecules. Fascinatingly, such a 'supermotif' was recently found in the class II-associated invariant chain peptide (CLIP), indicating that CLIP is a universal class II ligand in its interaction with the class II αβ dimer (Malcherek et al. 1995; Sette et al. 1995).

Class II molecules appear to alter their structure upon peptide interaction, and there is direct evidence that peptide association is necessary for the characteristic stability of class II dimers (Sadegh Nasseri et al. 1992). Experiments also suggest that a substantial fraction of class II in the endosomal loading compartment, does not find usable peptides under normal conditions and may not reach the cell surface (Srinivasan et al. 1991). This is achieved by aggregation of the empty dimers, ultimately leading to class II destruction (Stern et al. 1992). Thus peptide binding appears to have two distinct effects; one is to contribute to dimer stability by interaction with portions of the α and β chains, and the other is to change the structure of the class II molecule to add to its intrinsic stability (Lanzavecchia et al. 1992).

B1.3.4 Presentation of endogenous peptides via the MHC class II pathway

With regard to the class II pathway, there are numerous observations of MHC class II presentation of peptides from 'endogenous' proteins (Nuchtern et al. 1990). This raises the question of whether there is a non-endosomal site of...
antigen processing and peptide binding to class II molecules, namely a pre-golgi compartment. Perhaps the most intriguing data relates to the ability of a strictly cytoplasmic antigen, the measles virus matrix protein, to be presented by class II (Jacobson et al. 1989), and several subsequent similar reports of class II presentation of cytoplasmic proteins. The site of peptide-class II association in these cases is unresolved, although some evidence points to at least one of these antigens moving to the endosomal system for association with class II (Malnati et al. 1992). The question in that case switches to the site of peptide generation and the pathway followed by antigen or peptide to reach the endosomal compartment. This illustrates the fact that the class-specific function of MHC molecules relates primarily to their preferred site of peptide loading, which only incompletely dictates the protein sources (exogenous/endogenous) of those peptides. Clearly, however, the two systems are not interchangeable, as illustrated in the case of the severe immunodeficient phenotype seen in MHC class II deficient individuals, who possess relatively intact expression of MHC class I (section B.2.1).

B.2 CONTROL OF MHC CLASS II EXPRESSION

B2.1 MHC CLASS II DEFICIENCY

In 1980 (Griscelli, 1980) a new syndrome was described in children with severe immunodeficiency, who appeared to possess a complete lack of MHC class II expression. Since then less than 50 well documented cases have been reported, all with a similarly severe phenotype. The syndrome is known as MHC class II deficiency or the bare lymphocyte syndrome (BLS). In addition, a reduced cell
surface expression of MHC class I molecules has been described in certain cases (Amigorena et al. 1995), but the significance of this remains unclear. This confirmed the important role of MHC gene products in immune defence mechanisms, since all these patients have abnormal cellular and humoral responses to specific antigens.

Subsequent studies showed that this disorder was due to a lack of synthesis of class II molecules, and a lack of mRNA for all of the different MHC class II isotypes (LisowskaGrospierre et al. 1985). Direct transcription assays confirmed that none of the class II genes was transcribed (Reith et al. 1988). Since the MHC class II genes are not deleted, the global lack of expression of the entire class II gene family suggested a general defect in the regulation of these genes. This implicated one or several trans-acting factors (acting on the class II promoter region) would be affected, and strongly suggested that identification of the genes responsible for this disease would lead to important regulators of MHC class II expression.

**B2.1.1 The class II promoter region**

Henceforth, extensive studies on MHC class II promoters show that these promoters contain well-defined cis-acting sequences, called the W, X, X2 and Y boxes (figure 1), which are all required for both tissue-specific and IFNγ-inducible expression. Nuclear factors that bind to these DNA motifs have been characterised, and several have been cloned (e.g. RFX) (Ting et al. 1993). The protein factors identified were all found to be ubiquitous, suggesting that they were unlikely to account for cell-specific and inducible expression.
Figure 1: MHC class H promoter region, indicating cis-acting sequences W, X, X2 and Y boxes, and nuclear factors binding to these DNA motifs.
Figure 1: MHC Class II gene promoter

- RFX
- RFX1
- RFX2
- RFX3
- RFX4
- RFX5

CIITA

X2BP

NF-Y

Upstream Translation
More functionally relevant information on the molecular mechanisms controlling MHC class II gene transcription has been obtained by the study of the MHC class II deficient cell lines. Cell fusion experiments between several different patient and \textit{in vitro} generated cell lines has demonstrated the existence of at least four different complementation groups (Benichou et al. 1991), implying that mutations in at least four different genes result in the same disease phenotype (table 1). One further experimentally generated mutant cell line defines a fifth complementation group, for which no patient has yet been identified (Gladstone et al. 1978; Seidl et al. 1992). Two main groups of patients emerged, one that exhibits a defect in RFX binding, and the other showing normal RFX binding (Stimac et al. 1991). The former group displays a complete lack of occupancy of the class II promoter \textit{in vivo} (Kara et al. 1991) suggesting that normal binding of RFX is required for occupancy of the X2 and Y boxes. In the second group the class II promoter region is normally occupied.

\textbf{B.2.1.2 The discovery of CIITA}

One of these complementation groups corresponds to the aforementioned group with normal RFX binding, and in this group Mach's laboratory used a genetic complementation approach (figure 2) to clone the class II transactivator (CIITA) gene (Steimle et al. 1993). They were able to fully correct the class II regulatory defect of cells from these patients with bare lymphocyte syndrome. Their results indicate that CIITA is an essential mediator of inducible class II expression and therefore a general controller of MHC class II gene expression (Figure 1).
Figure 2: Schematic representation of complementation cloning method (Steimle et al., 1993). Plasmid DNA prepared from the HLA class I positive B cell line Raji was transfected by electroporation into the mutant (class II deficient) B cell line RJ2.25. Class II expressing cells were selected and found to contain a predominant 4.5kb cDNA insert.
Figure 2: Genetic Complementation

Raji

Transformation

RJ2.25

SELECTION - antibiotic and CII specific monoclonal Ab

RJ2.25 +cDNA

Transformation

Plasmid Rescue

cDNA
Table 1: Phenotypical, biochemical and molecular defects of MHC class II regulatory mutants from BLS complementation groups A-E

<table>
<thead>
<tr>
<th>BLS Complementation Groups</th>
<th>Wild Type</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototypical BLS cell line</td>
<td>BLS-2</td>
<td>BLS-1</td>
<td>SJO</td>
<td>None</td>
<td>ABI</td>
<td></td>
</tr>
<tr>
<td>Number of unrelated families</td>
<td>5</td>
<td>13</td>
<td>3</td>
<td>None</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Prototypical in vitro mutant</td>
<td>RJ2.25</td>
<td>None</td>
<td>None</td>
<td>6.1.6</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MHC class II expression</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MHC class II promoter activity</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>RFX-binding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>Promoter occupancy in vivo</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>Genetic defect</td>
<td>CIITA</td>
<td>?</td>
<td>RFX5</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

nt = not tested

B.2.1.3 Functional role of CIITA

Since the initial description of CIITA, its importance in the control of class II expression has been confirmed and further defined. Human CIITA is a 4.5kb cDNA coding for a protein of 1130 amino acids (Figure 3). The transcription activation function is provided by the N-terminal acidic domain (a.a. 26-137), which is experimentally exchangeable with a heterologous viral transcription-activating domain (Zhou et al.1995). The specificity of CIITA for three major MHC class II genes, DR, DQ and DP, is mediated by its remaining C-terminal residues (a.a. 317-1130). CIITA cDNA clones isolated show alternative RNA splicing, with only one splice site combination able to restore class II gene expression (Riley et al.1995). CIITA directs its activity through the X box element, with the presence of CIITA leading to the formation of a higher order complex at
the X box region. CIITA itself contains a potent activation domain, supporting its role in class II transcription.

MHC class II genes are expressed constitutively in only a few cell types, but they can be induced in the majority of them, in particular by IFNγ. It has been shown that CIITA expression is controlled and induced by IFNγ and that the JAK1 protein tyrosine kinase activity is required to induce the expression of CIITA upon IFNγ stimulation (Steimle et al. 1994; Chang et al. 1994). This indicates that CIITA is part of the signalling cascade from the IFNγ receptor to the activation of class II genes. In addition, the expression of CIITA is sufficient to activate class II genes in the absence of IFNγ stimulation suggesting that CIITA is the major regulatory factor for the inducible expression of class II genes.

A recent development in the unfolding story further widens the role of CIITA in antigen presentation. Mutant HeLa cells, defective in the expression of MHC class II genes, invariant chain and HLA-DM genes were transfected with CIITA cDNA. All three genes were re-expressed, suggesting that CIITA is a global regulator for the expression of genes involved in antigen presentation (Chang et al. 1995). Incidentally, RFX5, which operates at a different level of transcriptional control from CIITA, has also been shown to be an essential regulator of HLA-DM genes (Kern et al. 1995).
Figure 3: Protein structure of CIITA. The acidic domain, the 3 stretches rich in proline/serine/threonine and the consensus sequence for an ATP/GTP binding cassette are indicated.
B.3 THE INVARIANT CHAIN

B.3.1 INTRODUCTION

The invariant chain (Ii) was first identified in 1978, as a common polypeptide chain in different MHC class II immunoprecipitates (Jones et al. 1978). It is so called because of its non-polymorphic nature. Class II molecules are composed of two MHC-encoded subunits, the α and β chain, which associate in the endoplasmic reticulum (ER) with Ii.

Initially Ii was postulated to play a part in the assembly of the MHC class II complex, and also in the transport of MHC class II and other cell proteins. Subsequently, the structure and function of the invariant chain has gradually been delineated, from its synthesis in the endoplasmic reticulum, to its association with newly synthesised MHC class II. By its association with the class II molecule, Ii inhibits binding of endogenously synthesised peptides and also provides a localisation signal for the whole complex to be directed via the golgi apparatus to the endosomes. At this point the class II is proteolytically cleaved from Ii, so that exogenous peptides present in the endosomes can bind to the class II molecule, and thus be transported to the cell surface, for presentation to the appropriate T cells.

By this means Ii protects the newly synthesised class II molecule from acquiring endogenous peptide in the ER (thereby distinguishing MHC class II from the MHC class I molecule), and directs the class II molecule to cellular compartments where it can acquire exogenous peptides. Association with invariant chain thus determines the function of MHC class II molecules in antigen presentation.
B.3.2 INVARIANT CHAIN SYNTHESIS AND STRUCTURE

B.3.2.1 The invariant chain gene

The genes for MHC class II are regulated in a complex manner, being constitutively expressed, inducibly expressed, or not expressed depending on the cell type. However, the regulation and tissue distribution of the invariant chain is far less clear. There are clear similarities in the promoter regions of class II and Ii, and also in the interferon γ inducibility of the two molecules, but there is also evidence that regulation of the two is distinctive, which would have implications for the processing and presentation of peptide antigens in various cell types, under various conditions.

As far back as 1983 it was reported that the gene for Ii is not linked to the murine MHC class II (H-2) complex, thus showing that intracellular class II antigens consist of the products of two linked genes and one unlinked gene (Day et al. 1983). The human Ii gene has subsequently been mapped to band 5q32 (Genuardi et al. 1983) (while the MHC complex region is on the short arm of chromosome 6). The mouse Ii gene is located on chromosome 18 in the mouse (Strubin et al. 1986). The human gene was isolated in 1986, and consists of 8 exons spanning approximately 12 kilobases of DNA. The gene appears to exist as a single copy per haploid genome, and has none of the features of the immunoglobulin superfamily of genes (to which the α and β chains of the class II gene belong). This suggests that the evolutionary origins and also the function if the Ii gene is distinct from the MHC class II genes. Despite the unrelatedness of the structural component of these genes, consensus sequences found approximately 150 base pairs upstream
from the class II α and β chain genes were also found in analogous positions in the
Ii gene suggesting a possible role in the co-regulation of expression of these genes
(O'Sullivan et al. 1986). The 5’ regulatory sequences of the invariant chain gene
appear to be a combination of conserved class II regulatory elements and
promoter elements commonly found in other eukaryotic genes. Contained within
the promoter of the Ii gene are sequences (X and Igammat) that are similar to the
X and Y box elements of the class II gene promoter, suggesting that these
sequences might be involved in its regulation and contribute to the co-expression
of MHC class II and Ii genes (Zhu et al. 1990). This was confirmed when it was
shown that sequences homologous to the class II W, X and Y elements are present
in the promoter region of the Ii gene, and that interferon-gamma (IFN γ) inducible
expression of Ii in a cell line is regulated via these sequences (Brown et al. 1991a).
Interestingly, it has very recently been shown that CIITA, a MHC class II
transactivating factor which binds to the X box binding transcription factors (see
section B.2 for full review), is also required for expression of the invariant chain
(Chang et al. 1995), presumably binding to homologous transcription proteins that
bind to the X box element on the Ii gene.

B.3.2.2 Regulation and induction of invariant chain synthesis
As far as induction of Ii synthesis by cytokines is concerned, there is evidence that
similar structural features to the class II gene are present in the Ii gene, which
confer both IFNγ and tumour necrosis factor α (TNFα) inducibility. Nevertheless,
inducibility of the two genes is not entirely synchronous. In murine T-dependent
mast cells, for example, IFNγ induced both Ia (MHC class II) antigens and Ii
simultaneously. Also, variant lines of these cells failed to express either Ia or Ii upon IFNγ stimulation. However, B lymphocyte lines exposed to IFNγ enhanced the synthesis of Ii in the absence of Ia upregulation, indicating that in some cell lines Ii can be regulated independently of MHC class II expression (Koch et al. 1984). In human dermal fibroblasts and vascular endothelial cells, IFNγ induces Ii simultaneously with several MHC class II antigens (Collins et al. 1984). In a human colon carcinoma cell line which constitutively expresses neither MHC class II or Ii, administration of TNFα or IFNγ alone had no effect, but the cytokines together induced class II and Ii, with Ii mRNA detectable 10-12 hours after stimulation (Pessara et al. 1988). In a study of mouse tissue expression, upon IFNγ stimulation, MHC antigen expression was dramatically increased throughout the body, with striking differences in the inducibility of certain tissues for class II and Ii (Momburg et al. 1986).

A series of positive control elements, the kappaB element, Sp 1-b binding site, and CCAAT box are present in the Ii promoter, apparently serving distinct regulatory functions, for example in different cell lines (Doyle et al. 1990). This suggests that a sequence match between enhancers and certain promoter elements is important in Ii expression. An analogous mechanism has recently been demonstrated in a study of HLA-DRA expression on B cells and on HeLa cells. It concludes that distinct sets of transcription factors (binding to multiple sites on the promoter) are involved in HLA-DRA expression in the two different cell lines (Abdulkadir et al. 1995).

Thus a mechanism exists for differential expression of class II in different cells controlled via its promoter region, and similarly also for Ii. Also it is apparent that
despite the similarities in their promoter regions, Ii and class II do not necessarily respond in the same way in various cell lines to cytokine induction.

**B.3.2.3 Structure of the invariant chain**

A number of forms of invariant chain exist, defined by the primary amino acid sequence (figure 1). The first to be described were the p33 and p35 forms, resulting from the alternative use of two in-phase AUG initiation codons on a unique mRNA (Strubin et al. 1986), a mechanism only previously seen in viral systems. Soon after this, two additional forms of human Ii were described, p41 and p43 (O'Sullivan et al. 1987). p41 results from splicing of an additional exon and encodes a cysteine-rich stretch of 64 amino acids near the COOH terminus that displays a striking homology to an internal amino acid repeat of thyroglobulin, suggesting an evolutionary mechanism of exon shuffling. p33 is the major form of Ii, p35 does not exist in mice. The reason for the different forms of Ii is unclear. p35 and p43 share an N-terminal ER retention signal, and oligomers containing only the p33 or p41 forms of Ii can exit the ER. It requires class II to be co-expressed with p33 and p35 or the alternatively spliced p41 and p43, for the whole complex to be efficiently transported to the endocytic pathway (Arunachalam et al. 1994). Proteolysis of Ii has yielded two smaller products, p25, a non-membrane bound form of Ii formed at an early stage of Ii synthesis in the ER, and p28, a fragment generated under acidic conditions similar to those found in the endosomal compartment (Mehringer et al. 1991).
Figure 1: Protein structure of the human invariant chain indicating different isoforms, formed by alternate splicing. Arrows indicate N-linked glycosylation sites. CLIP = class II loading invariant chain peptide.
Figure 1

Structure of human invariant chain

- p33
  - NH2
  - COOH

- p35
  - NH2
  - COOH

- p41
  - NH2
  - COOH

- p43
  - NH2
  - COOH

TM | CLIP

29 56 81 104 | 216

amino acid length
B.3.3 MHC CLASS II TRANSPORT THROUGH THE CELL

B.3.3.1 Association of Ii with MHC class II in the endoplasmic reticulum

In the absence of association with class II glycoproteins, Ii is trimeric (Marks et al. 1990). Class II molecules on the surface of cells exist as a heterodimer of α and β subunits. The membrane-distal domains of these subunits form a peptide binding site consisting of a platform of eight strands of β-pleated sheet, with two α-helices forming the boundaries. In the ER, the invariant chain associates with the newly synthesised class II dimer via its extracytoplasmic (C-terminal) region (Marks et al. 1986). This complex has been demonstrated to be a nine-subunit transmembrane protein that contains three αβ dimers associated with an Ii trimer (Roche et al. 1991b) (figure 2). The association of Ii with class II is rapid and efficient, due to a large molar excess if Ii in cells expressing class II molecules (Kvist et al. 1982; Machamer et al. 1995). In mice bearing a deletion of the Ii gene, newly synthesised MHC molecules are largely misfolded, and fail to leave the ER due to the lack of the Ii transport signal (Elliott et al. 1994).

B.3.3.2 Inhibition of peptide binding to MHC class II in the ER

Purified class II αβ dimers can specifically bind antigenic peptides in vitro (Babbitt et al. 1995). Ii association with class II in the ER was first shown to inhibit immunogenic peptide binding utilising a well characterised influenza haemagglutinin-derived peptide, binding to HLA-DR only in the absence of Ii (Roche et al. 1990). Furthermore, HLA-DR/Ii complexes do not bear peptides recognised by alloreactive T cells, supporting this notion that class II/Ii association
prevents premature peptide loading (Demotz.1993). A short, 25 residue, contiguous internal segment of Ii frequently found associated with purified class II molecules has been designated the CLIP (class II associated invariant chain peptide) region and described as a nested set of class II associated peptides. This region is critical for class II folding, ER to golgi transport and inhibition of peptide binding (Romagnoli et al.1994). The CLIP region has been mapped to Ii exon 3, which encodes amino acids 82-107 (Sette et al.1995). CLIPs appear to bind in the class II peptide binding groove, and overcome allele-specificity by taking advantage of one or more supermotifs (Malcherek et al.1995). Proteolysis of the invariant chain in the complex with cathepsin B releases αβ dimers that bind antigenic peptides extremely well (Roche et al.1991b).

B.3.3.3 Transport of the class II/Ii complex to the endosomes

The intracellular transport of the classII/Ii complex was first elucidated in 1990 when it was shown that three structural motifs control the transport to endosomes from the ER (Lotteau et al.1990). An endoplasmic retention signal is present in the full length Ii. A truncated form of Ii is directed by another signal motif to a degradation compartment by a pathway circumventing the golgi. However, if the truncated Ii has the αβ dimers bound to it, a third signal dominates, directing the whole complex by way of the golgi to endosomes. This third endosomal localisation signal has subsequently been found to be a cytoplasmic domain of Ii (Roche et al.1992). Ii targets class II molecules to an acidic endosomal compartment, which is the site where class II molecules interact with the peptide to be presented (Lamb et al.1991). Upon further analysis, the route of class II
transport to the site of antigen processing and loading involves movement through early endosomes to late endosomes, the rate of which is determined by the level of Ii expression (Romagnoli et al.1993). In Ii deletion experiments, class II dimers appear misfolded and are inefficiently transported from the ER to the golgi. In addition, class II transported through the golgi accumulates an abnormally increased molecular mass associated with N-linked glycosylation (Anderson et al.1992). Thus Ii appears to act as a class II specific chaperone in its transport through the cell.

B.3.3.4 Dissociation of the MHC class II/Invariant chain complex

Once the class II/Ii complex is present in the endosome, it needs to be degraded proteolytically (Roche et al.1991a) in order for both loading of exogenous peptides to the class II dimer and for transport of class II to the cell surface. It has been shown that dissociation of Ii from class II is required for transport of the αβ dimer to the cell surface (Loss, Jr. et al.1993). Class II molecules appear to traffic through most of the endocytic pathway, permitting capture of distinct determinants made available under differing conditions of pH and proteolytic activity (Castellino et al.1995; Urban et al.1994). Most recently it has been found that in B lymphocytes there is a novel population of endocytic vesicles involved in the transport of MHC class II/peptide complexes to the cell surface. These vesicles are likely to serve as a specialised site, distinct from endosomes and lysosomes, that host the final steps in the dissociation of Ii from class II molecules and the loading of antigen-derived peptides onto αβ dimers (Amigorena et al.1995).
Figure 2

Schematic representation of Invariant chain structure
B.3.4. FUNCTIONAL CONTROL OF THE INVARIANT CHAIN

B.3.4.1 A human class II related locus, HLA-DM

In 1991 a previously undiscovered class II related protein was described (Zemmour et al. 1993). The genes, DMA and DMB, map between the HLA-DNA and -DOB loci, and encode \( \alpha \) and \( \beta \) chains of this novel member of the immunoglobulin gene family. Recently it has been found that HLA-DM catalyses the dissociation of class II associated invariant chain peptide from MHC class II-CLIP complexes in endosomes, at an acidic pH optimum, and facilitates the binding of antigenic peptides (Denzin et al. 1995). Furthermore, X-ray crystal structure determination shows that the CLIP fragment binds to HLA-DR3 in a way almost identical to that in which antigenic peptides bind class II glycoproteins. Hence, this structure is the substrate for the loading of antigenic peptides by an exchange process catalysed by HLA-DM (Sloan et al. 1995). The essential role of this molecule is illustrated by various cell lines deficient in antigen presentation, which have been shown to lack HLA-DM (Morris et al. 1994).

B.3.4.2 An alternative route for endosomal localisation

There is also evidence of an alternative pathway of \( \iota/ \)class II routing that directs the complex directly from the ER to the cell surface, initially. As mentioned, the cytosolic tail of \( \iota \) contains the endosomal localisation signal that directs newly synthesised class II to within the endosomal antigen processing compartment. Deletion of this signal results in rapid transport of class II/\( \iota \) complexes to the cell surface, from where it is efficiently internalised to endosomes, followed by
degradation and return of free class II to the cell surface. Interestingly, the efficiency of antigen presentation to T-cells is preserved in these cells, suggesting that endocytosed class II can form immunogenic complexes with newly processed antigen (Anderson et al. 1993; Nijenhuis et al. 1994). This process can occur physiologically as seen in class II negative cell lines, wherein Ii enters the endocytic route via the cell surface (Henne et al. 1995), or in a B cell line in which a large population of class II/Ii complexes are seen to reach endosomes by rapid internalisation from the cell surface (Roche et al. 1993).

B.3.5 THE ROLES OF MHC CLASS II AND INVARINAT CHAIN EXPRESSION

B.3.5.1 Cell surface expression of Ii and of MHC class H molecules

From the endosomes, the newly formed class II/exogenous peptide complex travels to the cell surface by a process that is incompletely understood. It is unclear what proportion of class II/Ii complex or, indeed, free Ii is transported to the cell surface, or what purpose this would serve in the efficiency of antigen presentation. The invariant chain can be expressed on the cell surface, as demonstrated by monoclonal antibody localisation (Wraight et al. 1990). On a B cell line, immunohoehemical studies reveal rapid surface expression of Ii, a proportion of which is associated with class II antigen (Koch et al. 1991b). However, the presence of Ii is not necessary for MHC class II cell surface expression, which gave an early indication that Ii did not simply mediate membrane expression of class II
molecules (Sekaly et al. 1986; Miller et al. 1986), although mice lacking Ii expression do show a dramatic reduction in surface class II (Bikoff et al. 1993). Confusingly, Ii deficient fibroblasts, although expressing an altered form of class II at the cell surface, have been shown to present antigenic peptide to T cells as efficiently as Ii expressing cells (Sekaly et al. 1988; Peterson et al. 1990). There is contrasting data suggesting that high Ii levels correlate with an increased ability of fibroblasts to present exogenous antigen (Bertolino et al. 1991). To support the former, cells expressing surface class II/Ii complexes interact with staphylococcal toxins and stimulate polyclonal T-cells as efficiently as cells expressing only class II at the cell surface (Karp et al. 1992). To support the latter, fibroblast transfectants that fail to dissociate Ii from class II, hence expressing mainly class II/Ii complexes at the cell surface, are inefficient in their ability to present peptides to T-cells (Roche et al. 1992).

Conversely, human B cells expressing at least one class II isotype express normal amounts of Ii on the cell surface, but total class II deletion results in diminished expression of Ii. Also, unexpectedly, Ii is not synthesised on de-novo induced class II positive T-cell clones (Wilson et al. 1993b).

The cell surface expression of MHC class II is thus not clearly dependent on the invariant chain, although there is a suggestion that Ii expression is attenuated in the absence of class II. Also there is contrasting evidence concerning the role of Ii expression (alone or with class II) at the cell surface.
B.3.5.2 Consequences for antigen presentation

As alluded to above, the role for li in class II restricted antigen presentation has been controversial. There was initially indirect evidence for the importance of li, wherein fresh epidermal Langerhans cells (LC) synthesised higher amounts of li and were far more efficient at antigen presentation than cultured LC's (Pure et al. 1990). This evidence strengthened with the finding that antigen presentation is facilitated by the p41 form of li (Peterson et al. 1992). Furthermore, the class II conformation change ascribed to li binding is retained after li dissociates. This altered conformation affects recognition by allogeneic T-cells, which preferentially recognise class II molecules that have been associated with li (Rath et al. 1992). A dramatic finding in this respect concerns the small proportion of li that is modified by the addition of chondroitin sulphate (li-CS). This form of li is expressed at the surface of antigen presentation cells, and greatly enhances the ability of the cell to stimulate T-cells, by means of li-CS functioning as an accessory molecule, interacting with CD44 on the T-cell (Naujokas et al. 1993). Finally, li deficient mice present exogenous antigen very poorly, and are deficient in producing and negatively selecting CD4+ T-cells (Viville et al. 1993). Thus, the bulk of the evidence so far points towards a crucial role for the li/class II interaction in the ultimate presentation of immunogenic antigen, and which is also likely to play a part in selecting the particular peptide epitope presented (Bodmer et al. 1994).

B.3.5.3 The role of MHC class II distribution in antigen presentation

Class II molecules, although usually found on 'professional' antigen presenting cells such as B cells and macrophages, can also be found on endothelial cells, and
constitutively on epithelial cells of only a small number of tissues (e.g. thymus, small intestine, renal proximal tubule). From classical teaching, the true immunologic periphery was only thought to constitute satellite lymphoid tissue, abundant in professional antigen presenting cells. It was eventually realised, however, that macrophages and dendritic cells are also scattered throughout parenchymal organs, like thyroid, liver and kidney (Halloran et al. 1985; Bottazzo et al. 1986; Hart et al. 1981a) and that perhaps, auto-immune and allogeneic responses could be established locally at the site of emerging inflammation. It has subsequently been demonstrated that non-lymphoid somatic cells are also capable of aberrantly increasing the numbers of MHC class II molecules on their cell surface (Halloran et al. 1985; Bottazzo et al. 1986), probably following cytokine exposure in their local microenvironment (Haverty et al. 1989; Glimcher et al. 1992). This leads to the hypothesis that parenchymal cells, like epithelium, might also be able to process and present antigenic peptides to T lymphocytes (Bottazzo et al. 1986; Haverty et al. 1988). The role of class II on epithelial cells is thus, incompletely understood. Chondrocytes and enterocytes for example, can process and present antigen to T cells, but other non-haematopoietic cells such as keratinocytes and pancreatic β cells induce antigen specific unresponsiveness in T cells (Hines et al. 1989). Renal tubular epithelial cells, uniquely among the cells of transplanted organs, express MHC class II constitutively. They are speculated to participate in the pathogenesis of immune renal injury, and have been shown to present both self and foreign antigen (Hart et al. 1981a), although their role in allograft rejection is unclear (Hagerty et al. 1992).
B.3.5.4 The role of invariant chain distribution in antigen presentation

The expression of li on B cells may be high or low, depending on the class II haplotype, and class II negative B cell lines show low levels of li expression (Wilson et al. 1993a). Dendritic cells, which are potent antigen presenting cells and are present in the connective tissues of many organs (Hart et al. 1981b), show a rapid turnover of li in culture, compared to a slow class II turnover (Pure et al. 1990), implying distinct regulation of the two. The expression of li on non-haematopoietic cells is less clear. Vidal et al. report that murine gut epithelial cells express class II but are deficient in li, suggesting an altered conformation of class II on enterocytes, which could affect the nature and presentation of peptides. In a study of normal colorectal mucosa, adenomas and carcinomas, li was expressed much more strongly than class II, particularly in the diseased cells (Momburg et al. 1988b). The variability of cell type expression is further illustrated in a study of ileum affected by Crohn’s disease. Corresponding to local inflammation, a considerable neo-expression of class II antigens and li was observed in epithelial cells and arterial endothelial cells. However, the picture was different in normal ileum, where in crypt enterocytes and vascular endothelial cells, li was found in the absence of class II expression. In contrast, in class II positive venous and capillary endothelial cells li was greatly reduced or absent (Momburg et al. 1988a). Physiological li expression on other tissues has not been systematically studied, particularly with reference to MHC class II expression.
B.4 THE T-CELL AND CYTOKINE RESPONSE IN ANTIGEN PRESENTATION

B.4.1 INTRODUCTION

During the last few years, researchers have begun to dissect the complicated cascade of events that are required for T-cell activation during antigen presentation. At least three stages can be identified. First, non-cognate adhesion occurs between T-cells and potential antigen presenting cells through the molecular interaction of cell surface receptor-ligand pairs such as endothelial leucocyte adhesion molecule (ELAM-1) to E-selectin, or very late activation antigen (VLA-4) to vascular adhesion molecule (VCAM-1), or lymphocyte function associated antigen (LFA-1) to intercellular adhesion molecule (ICAM-1). These early events are followed by a cognate interaction between the T-cell receptor αβ heterodimers and their co-receptors, CD4 and CD8, with the MHC-peptide complex. Finally, a second set of non-cognate interactions between cell surface accessory molecules results in essential co-stimulatory signals required for a complete T-cell response (figure 1). The interruption of T-cell activation at any one of these stages may result in the suppression of graft rejection, and possibly the development of donor-specific tolerance.
B.4.2 ACCESSORY MOLECULES INVOLVED IN T-CELL ACTIVATION

Occupancy of the T-cell receptor (TCR) by processed antigen/MHC provides the first, but not totally sufficient signal for full T-cell activation. Signal two, or 'co-stimulation' is derived from ligand-ligand interactions between the surfaces of APC's and T-cells (Janeway, Jr. et al. 1994). All APC's (e.g. dendritic cells, activated macrophages or B-cells) are able to stimulate primed T-cells, whereas dendritic cells are the most potent APC's in providing these co-stimulating signals to the naive T-cell (Macatonia et al. 1993).

Key cell surface molecules involved in this process are CD28 (June et al. 1990) and cytotoxic T lymphocyte associated antigen (CTLA)-4 (Linsley et al. 1991) expressed by T-cells interacting with the B7/BB1 molecule expressed by APC's and activated B cells. Other accessory molecules expressed by APC's, such as ICAM-1 and LFA-3 may also have important co-stimulatory functions (Damle et al. 1992). Both alloreactive CD4 (Koulova et al. 1991) and CD8 (Harding et al. 1993) T-cells can be activated as a consequence of the CD28-B7 interaction. Once activated, CD4 T-cells execute a carefully orchestrated pattern of cytokine production, which is essential for achieving T-cell dependent immune phenomena. Accessory molecules involved in the interaction between T-cells and APC's (figure 1) have provided much promise for specific manipulation strategies.
Figure 1: Receptor-ligand pairs between activated T-cells and APC’s (Boussiotis et al., 1994)

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<th>T-cell - APC interactions</th>
<th>Stages of T-cell response</th>
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<td>LFA-1</td>
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Adhesion Immunosuppression
Co-stimulation Anergy
B.4.3 THE B7-CD28/CTLA4 PATHWAY

B.4.3.1 Ligand and receptor characteristics

To date, two CD28/CTLA4 counter-receptors have been cloned and functionally characterised, termed B7-1 and B7-2 (Hathcock et al. 1994). These molecules, which can be induced on a wide variety of APC's (Boussiotis et al. 1993) are members of the immunoglobulin gene superfamily (Freeman et al. 1993b). Although B7-1 and B7-2 demonstrate only 25% amino acid homology, they are both low-affinity receptors for CD28 and high affinity receptors for CTLA-4 (Linsley et al. 1993). Some investigators have shown B7-1 and B7-2 to be constitutively expressed on dendritic cells (Young et al. 1992), but other studies find B7-1 expression to be primarily induced (Hart et al. 1993).

B7-2 is also constitutively expressed on monocytes (Azuma et al. 1993) but most other APC's require some stimuli to induce their expression.

In spite of their structural differences, both B7-1 and B7-2 signal via the same T-cell receptor, CD28. In humans, CD28 is constitutively expressed on 95% of resting CD8 peripheral blood T-cells and its expression increases following activation (Linsley et al. 1993). A second receptor on T-cells for the B-7 family members is CTLA4 (Brunet et al. 1987). Although this molecule is 31% identical to CD28 at the amino acid level, it is not expressed on resting T-cells (Linsley et al. 1992) and is induced following T-cell activation. Interestingly, the cytoplasmic domain of the CTLA4 molecule displays remarkable phylogenetic conservation (100% between human, mouse and chicken) (June et al. 1994), suggesting a conserved signalling function. Also, B7 family members have a higher affinity for
CTLA4 than for CD28, so it is not surprising that an immunoglobulin fusion protein of CTLA4 (CTLA4 Ig) has proven to be the most effective reagent to inhibit B7 family co-stimulatory function both in vitro and in vivo (Lenschow et al. 1992).

B.4.3.2 Studies on the role of B7-1 and B7-2

Three model systems have addressed the functional role of the counter-receptors for CTLA4/CD28. The B7-1 deficient mouse provided evidence for the existence of more than one counter-receptor (Freeman et al. 1993a) wherein activated B-cells expressed the alternative receptor, B7-2, leaving the pathway intact. Secondly, B7-1 transgenic mice (Sethna et al. 1994), constitutively expressing the B7-1 gene on mature B-cells, have greatly reduced levels of serum immunoglobulins. In addition, these mice demonstrate reduced antibody responses to T-dependent hapten-protein conjugates, which is restored by the administration of anti-B7-1 mAb. This suggests that the normally regulated B7-1 response may contribute to either the initiation or the down-regulation of a T-dependent B-cell response. Finally, T-cells from CTLA4 Ig transgenic mice could not deliver B-cell help, as measured by impaired antibody function (Lane et al. 1994), implying impaired T-B-cell interactions in the presence of CTLA4 Ig.

B.4.3.3 The roles of CD28 and IL-2 in the T-cell response

Following a TCR-mediated signal, ligation of CD28 results in up-regulation of IL-2 receptor (IL-2R)-γ and IL2R-α chains (Cerdan et al. 1992), increased IL-2 mRNA transcription (Fraser et al. 1992), cytokine secretion, T-cell proliferation (Freeman
et al. 1993b), up-regulation of CD40 ligand (De Boer et al. 1993), and up-regulation of CTLA4 mRNA (Lindsten et al. 1993).

CD28 deficient mice, perhaps surprisingly, have normal T-cell development although helper T-cell activity is significantly reduced. In addition, antibody secretion and immunoglobulin class switch after viral antigenic stimulation is significantly decreased, as is IL2R signal up-regulation along with proliferation and IL-2 secretion in response to mitogens (Shahinian et al. 1993). However, T-cells from these mice appear to have normal cytolytic activity, suggesting that an alternative co-stimulatory pathway might induce the production of cytokines. IL-2 deficient mice also have normal thymocyte and peripheral T-cell subsets, reduced but inducible polyclonal T-cell responses, and reduced levels of several immunoglobulin subtypes (Schorle et al. 1991). Like the CD28 knockout mice, they have relatively normal cell-mediated immune responses. These data suggest that the major function of CD28 and of IL-2 is the induction of T-cell help for humoral immunity. Moreover, T-cells from either of the deficient mouse strains described are not anergic, consistent with the hypothesis that in the absence of IL-2 or CD28, an alternative signal (possibly delivered by IL-4 or IL-15) might replace those signals in the prevention of anergy. The recent observation that a subtype of X-linked severe combined immunodeficiency (SCID) in humans is the result of mutation of the common gamma chain of the IL-2, IL-4 and IL-7 receptors (Noguchi et al. 1993) argues for the functional redundancy of the IL-2, IL-4 and IL-7 receptor signaling.
B.4.4 THE T-CELL RESPONSE

B.4.4.1 T-cell activation

Occupancy of the T-cell receptor by processed antigen/MHC complex is followed by 'signal two' or co-stimulation, as described above. Several groups have found that dendritic cells are the most potent APC's in providing these co-stimulation signals to the naive T-cell e.g. (Macatonia et al. 1993), whereas all APC's are able to stimulate primed T-cells. Once activated, CD4 T-cells execute a carefully orchestrated pattern of sequential de novo gene expression and cytokine production, which is essential for achieving T-cell dependent immune phenomena.

B.4.4.2 The Th1/Th2 model of CD4 cell activation

With the realisation that individual CD4 T-cell clones express phenotypically distinct cytokine profiles, a unifying concept, the Th1/Th2 paradigm, has evolved (figure 2). Activation of the T helper (Th1) phenotype is strongly associated with cell mediated immunity, whereas the Th2 phenotype is often associated with humoral immunity. Individual cytokines are viewed as regulators of Th1/Th2 cell function. Activated CD4 cells have been categorised and subclassified on the basis of their phenotypic pattern of cytokine production: Th0 cells (IL-2, IL-4, IFNγ, lymphotoxin), Th1 cells (IL-2, IFNγ, lymphotoxin), and Th2 cells (IL-4, IL-5, IL-6, IL-10) (Street et al. 1991).

Using transgenic mice, with the IL-4 promoter upstream of the gene for herpes simplex virus (rendering IL-4 producing T-cells susceptible to ganciclovir), the role of IL-4 producing T-cells has been studied. Naive T-cells stimulated in vitro in the
presence of IL-12 or IL-4 become IFN\(\gamma\) or IL-4 producers respectively. Exposure of the naive T-cells to ganciclovir eliminates production of both IFN\(\gamma\) and IL-4. Thus, effector cells producing IL-4 or IFN\(\gamma\) probably differentiate from a common IL-4 producing precursor (i.e. the Th0 cell) (Kamogawa et al. 1993). That the Th1 and Th2 phenotype evolve from a Th0 phenotype is indirectly supported by data showing that naive T-cells which become IL-4 producers require exposure to IL-2, in addition to IL-4, in order to acquire the Th2 phenotype (Seder et al. 1994). Taken together, these studies support the concept that naive T-cells are not pre-committed to a Th1 or Th2 phenotype, but the phenotype varies with the cytokine micro-environment. Furthermore, Th1 and Th2 cells develop from a common precursor and may cross-regulate one another by the release of their respective cytokines (IFN\(\gamma\) and IL-4) (Fitch et al. 1993). Hence, activation of the Th1 phenotype is often accompanied by silencing of the Th2 pathway, and vice versa.

B.4.5 CYTOKINE ACTIVATION IN ALLOGRAFT REJECTION

Acute allograft rejection is a T-cell dependent process, and CD4 cells play a central role in the rejection process in normal mice (Hall. 1991). Proteins and/or transcripts for intragraft IL-2, IFN\(\gamma\), and the CTL specific marker, granzyme B, but not IL-4, have consistently been found in rejecting allografts (Dallman et al. 1991a). Moreover, sequential analysis of rejecting experimental allografts has consistently detected IL-2 and IFN\(\gamma\) expression preceding or accompanying graft dysfunction. IFN\(\gamma\) is believed to recruit macrophages into the graft, cause macrophage activation, enhance CTL activation, and promote enhanced MHC expression by the graft. IL-2, principally a product of CD4 cells, stimulates proliferation of CD4 cells
in an autocrine fashion and leads to the paracrine activation of CD8 CTL’s. Thus the expression of IFNγ and IL-2 are closely linked to the activation of cellular proinflammatory immune responses.

IL-2 expression is known to be strongly associated with rejection and it has been demonstrated that IL-2 administration, as an adjunct to tolerising therapies, precludes engraftment (Dallman et al. 1991b). It is not clear, however, whether the absence of IL-2 expression causes tolerance. In the IL-2 knockout mouse, where it would be expected that the Th2 phenotype (closely associated with transplantation tolerance) is preferred over the Th1 phenotype (requiring IL-2), allografts are in fact readily rejected (Schorle et al. 1991). This implies that more than a single T-cell growth factor can support proliferation of alloreactive T-cells and rejection. Perhaps a more illuminating experiment supporting this notion, is the creation of a transgenic mouse in which the IL-2 promoter was placed upstream of the gene for HSV thymidine kinase (Minasi et al. 1993). Thus T-cells expressing IL-2 are rendered susceptible to ganciclovir, and the immune system can be depleted of IL-2 producing cells at any stage of T-cell activation. This demonstrated that superantigen induced T-cell activation is IL-2 dependent, whereas concanavalin A induced proliferation is only partially mediated by IL-2, supporting the notion that other growth factors are involved.
Figure 2: CD4 T-cell activation, the Th1/Th2 paradigm
B.4.6 THE SEARCH FOR TRANSPLANTATION TOLERANCE

B.4.6.1 Cytokine mediated transplantation tolerance

The possibility that disruption of the cytokine network may provide a new approach to regulation of the immune response to allografts, is a considerable spur to a large body of work studying the complex cytokine interactions that occur following transplantation.

It has become clear over the past few years that blockade of the IL-2 pathway may result from (Dallman et al. 1991b), and indeed be responsible for (KupiecWeglinski et al. 1988) the development of tolerance in experimental models. This idea has been extended to suggest that a block in Th1 type cells, the primary source of cytokines such as IL-2 and IFNγ, is associated with the induction of tolerance (e.g. Bugeon et al. 1992). It has been shown that anergic donor-specific cells that have reduced ability to produce IL-2 may also exist in patients following transplantation. Upon stimulation with alloantigen, cells from patients who displayed hyporesponsiveness towards donor antigens were seen to express IL-2 receptor, but did not proliferate, or make IL-2 (Alard et al. 1993). This result resembles previous studies in rat models of tolerance, and strongly suggests that donor-reactive cells are present, but anergic, in hyporesponsive individuals.

There is some evidence that the expression of certain cytokines, associated with Th2 cells (e.g. IL-4) (Gorczynski. 1992), may be preserved in tolerant animals, although the presence of such cytokines does not necessarily confirm the presence or action of Th2 cells. The observed increase in IL-4 production within accepted heart allografts in rats (Papp et al. 1992), and reduced IL-2 production in
established renal allografts in rats (Dallman et al. 1991a), supports a role for relative Th subset responses in graft acceptance. Since IFNγ, which is produced by Th1 cells, upregulates graft MHC expression (Hao et al. 1990) and augments DTH, it would be expected that a Th2 type response would suppress graft rejecting Th1 type cells. This is one currently envisaged scenario for antigen-specific tolerance. The immune response to transplants is far from straightforward, and it has become clear that grafts may be rejected by either cellular or humoral means. Therefore, it may also be possible that shifting the immune response away from a cell mediated to a humoral type of response will not lead to tolerance but merely to rejection through an alternative mechanism.

**B.4.6.2 Anergy as a means for tolerance**

Anergy in T-cells was first described by Lamb, who showed that T-cell clones exposed to antigen on other T-cells in the culture responded in such a way that subsequent exposure of these T-cells to antigen on normal APC’s did not result in T-cell proliferation (O’Hehir et al. 1990). This functional unresponsiveness was considered similar to the clonal anergy described for B-cells (Pike et al. 1982). T-cell anergy can result from antigen receptor stimulation in the absence of co-stimulatory signals, as described earlier. The precise mechanism for this is still very speculative. For example, it has been suggested that any stimulus promoting cell division can prevent anergy. In this model, the first signal leads to the formation of both positive and negative regulators of IL-2 transcription, while the second signal inhibits the negative regulators, allowing for IL-2 production (DeSilva et al. 1991).
The precise nature of the second signal is unclear, and may not be provided by only a single molecule or a single pathway, and also there is heterogeneity among T-cells as to second signal requirements. The CD28/B7 pathway described seems the best candidate to date, but inhibition of accessory molecules other than CD28 has also been shown to facilitate graft survival \textit{in vivo} as well as to suppress antibody responses long-term. Non-depleting anti LFA-1, anti ICAM-1 and anti VLA-4 treatments can inhibit graft rejection and may induce transplantation tolerance (Isobe et al.1992; Paul et al.1993). Despite the success of these anergy-inducing strategies in rodents, little success has yet been achieved in primates or humans. Therefore novel ways of inducing anergy, for example utilising the antisense oligonucleotide approach, are potentially valuable alternative approaches.

Anergy can be detected in long-term graft acceptance in a number of different transplantation models. The key to detecting anergy in these situations is the association of certain V\(\beta\) TCR families with reactivity to MHC antigen or to superantigens. For example, in mice, bone marrow transplantation across non-MHC barriers can be achieved by appropriate cell population administration (Rammensee et al.1989) and by monoclonal anti-T-cell antibody administration (Qin et al.1989). Long-term graft acceptance is accompanied by a mixing of host and donor cell types. When donor and host differ at the Mls 1\(^a\) (superantigen) locus, the fate of Mls 1\(^a\) reactive V\(\beta\)6 and V\(\beta\)8.1 T-cells can be monitored. When successful engraftment occurred in these models, V\(\beta\)6/ V\(\beta\)8.1 T-cells were present at near normal frequencies. Analysis of the reactivity of V\(\beta\)6 cells \textit{in vitro}
confirmed a proliferative unresponsiveness associated with reduced IL-2 production which could be overcome by the addition of IL-2.

Anergy as defined herein is a proliferative unresponsiveness, and it is confined to Th1 (IL-2 producing) CD4 T-cells. While this may be indicative of an inability to mediate graft rejection, proliferation and cytolytic potential are not necessarily synonymous. Thus proliferative anergy may not equate with lack of rejection capacity in all circumstances.

In the last few years, advances have been made on several fronts in the understanding and manipulation of allograft tolerance. Under appropriate conditions, inhibition of the B7-CD28 pathway can result in antigen specific tolerance _in vivo_, albeit as a transient and reversible state.

Collaboration between CD4 and CD8 cells in allograft immunity is probably more complex _in vivo_ than simply the consequence of IL-2 production for responsive T-cells. The APC, via MHC class II signalling, and local cytokine production, participate actively in determining the outcome of the response. The cytokine profiles suggest that selective Th1 activation is associated with rejection, and selective Th2 activation is associated with the induction of tolerance. This paradigm has provided a frame of reference to test various hypotheses, and the study of the IL-2 knockout mouse, for example, indicates that other events are also critical in tolerance induction. The role of IL-2 in determining the direction of the immune response remains crucial, however, and specific manipulation strategies are likely to prove useful in further understanding the process, and perhaps moving towards the tolerant state required in transplantation.
B.5 MECHANISMS OF ALLOGRAFT REJECTION

B.5.1 INTRODUCTION

The notion of histocompatibility genes was introduced by Snell in 1948, by studying the genes controlling skin graft rejection in mouse inbred strains (Snell.1948). They were initially described as transplantation antigens, long before there was any information on the antigens themselves. The discovery of the structure of the MHC molecules and the subsequent studies elucidating the function of the MHC in relation to antigen processing and presentation have made it possible to study in detail the molecular mechanisms of T-cell repertoire selection, and self-restricted T-cell recognition of foreign or nominal antigen.

In the immune system, there is considerable interdependence between different cell subpopulations. Cell-mediated immune mechanisms include T-cell mediated processes with high degrees of specificity, as well as more poorly restricted responses by natural killer cells, lymphokine activated killer cells, and macrophages. Humoral mediated responses can be natural antibodies or highly specific antibodies of a variety of isotypes. All these mechanisms have been implicated as effectors of allograft recognition. The principal targets of the immune response to allografts are the MHC molecules themselves, and T-cell recognition of allo-MHC is the primary and central event that initiates allograft rejection (Krensky et al.1990; Steinmuller.1985).
B.5.2 THE T-CELL RESPONSE TO ALLOGRAFTS

Understanding how T-cells recognise allo-MHC is key to understanding subsequent events leading to allograft rejection, and ultimately for the future development of specific immunotherapies. The critical role of T-cells in allograft rejection was first established by the failure of neonatally thymectomised (227) and congenitally athymic nude rodents to effect rejection (Corley et al. 1977). We now know that allorecognition involves a tripartite structure consisting of T cells, MHC molecule and a peptide bound in the groove of the MHC molecule (Eckels.1990). Recent evidence advocates the occurrence of at least two distinct, but not necessarily mutually exclusive, mechanisms of allorecognition, the ‘direct’ and ‘indirect’ pathways (figure 1).

B.5.3 THE ‘DIRECT’ PATHWAY OF ALLORECOGNITION

This has been the more traditional and well documented mode of allorecognition, in which T-cells recognise intact allo-MHC molecules on the surface of donor cells. It has long been recognised that the normal T-cell repertoire contains a high frequency (1-10%) of total T-cells that are capable of responding to allo-MHC molecules (Sherman et al.1993). This translates to a precursor frequency at least 100 times that of antigen-specific self-restricted T-cells.
Figure 1: Direct and indirect pathways of alloantigen presentation

**DIRECT**
- Donor APC
- Donor MHC
- TCR

**INDIRECT**
- Recipient APC
- Recipient MHC
- Donor MHC peptide

Recipient T-cells
B.5.3.1 Theories to explain direct recognition

The two fundamental questions in allore cognition are: First, why is the frequency of alloreactive T-cells so high? Secondly, how can positively selected, self MHC-restricted T-cells recognise foreign antigens as well as allo-MHC? Several hypotheses have been put forward by different groups of investigators (figure 2).

One of the earliest attempts to explain the basis for the high frequency of alloreactive cells was described by Jerne (Jerne.1971; van Boehmer et al.1978). This theory proposed that the repertoire of T-cell receptors was evolutionarily preselected to include germline genes that had specificity for the MHC molecules of the species. Thus, for each MHC molecule in the species, there evolved a complementary receptor molecule. This readily explained why a high percentage of T-cells respond to each MHC molecule. A prediction of this theory would be that the repertoire used in recognition of antigen was separate from the allospecific repertoire. However, there is now evidence from the study of T-cell clones, that the antigen specific and alloreactive T cell repertoires may be contained within the same clones (Braciale et al.1981; Sredni et al.1980; Hunig et al.1981), and that the same portion of the T-cell receptor (TCR) involved in the recognition of antigen plus MHC is involved in recognition of alloantigen (Matis et al.1987).

Matzinger and Bevan also developed a hypothesis that attempted to explain the high frequency of alloreactive cells (Matzinger et al.1977). They proposed that a single allo-MHC product can stimulate multiple T-cell clones by forming multiple ‘binary complexes’ with endogenous molecules. These complexes result from the association of numerous self antigens with allo-MHC molecules in the cell membrane. Allogeneic MHC molecules bind a different set of antigens (now known
to be peptides) to that selected by self-MHC, and those peptides that bind both self and allo-MHC products may bind to the allo-MHC molecule in a different orientation. This theory was based on the observation that cytotoxic T-lymphocytes (CTL) could be obtained between MHC identical strains that differed from each other in expression of minor antigens (Bevan. 1975). At the time, these antigens were believed to represent polymorphic cell surface proteins. Considering that hundreds of different proteins may be expressed by the cell and available for interaction with the MHC, the high frequency of alloreactivity was a consequence of the diversity of antigenic complexes available for recognition. In contrast to the theory of Jerne, this hypothesis permitted a high degree of TCR diversity within the alloreactive repertoire, as well as no conceptual restrictions on overlap between the allospecific and self MHC-restricted antigen specific repertoires, and has turned out in broad concept to be accurate.

Bevan, later, further proposed that, unlike conventional foreign antigens, which must compete with the multitude of cellular epitopes to interact with MHC for presentation to T-cells, and therefore would be found associated with relatively few class I molecules, the foreign sequences on an allo-MHC were present on all class I molecules and therefore were far more abundant (Bevan. 1984). This high concentration of antigen could permit stimulation of many T-cells of relatively low affinity, thus involving a higher proportion of the T-cell repertoire (the so-called 'high determinant density' hypothesis). In this case, bound peptides, if present, may not be of central importance. These theories may, therefore, provide an explanation of how T-cells that have been positively selected in the thymus for self MHC-restriction could recognise allo-MHC.
Figures 2a and 2b: Illustration of two predominant hypotheses for high frequency of alloreactive T-cells (Matzinger, 1977 and Bevan, 1984).
Figure 2a:

MHC class I

Donor cell

allogeneic peptide

Multiple binary complexes

T-cell
Figure 2b: Donor peptide

Donor cell

Class I molecule

High determinant density

T-cell
Lechler and Batchelor used results from studies of specific HLA-DR primed T-cell clones to propose that an allo-MHC can be thought of as having two functional sites (Lechler et al. 1991; Lechler et al. 1990; Lombardi et al. 1991; Lombardi et al. 1989). The first region is formed by the regions of the amino-terminal domains that contact the TCR. The second site is formed by the regions of the antigen-binding groove. Therefore, T-cell responses to alloantigen will depend on the similarities or differences between responder and stimulator MHC sites. In the case of similarities in the region of the TCR binding site, allorecognition would be due to host T-cells exhibiting sufficient affinity to recognise novel peptides bound by the allo-MHC molecules. In the case of differences in the region of TCR binding, the actual ligand may be the MHC molecule itself, the host T-cell binding to an allo-MHC molecule to which it has by chance a 'better fit', and the bound peptide may not play a significant role. This latter model would be compatible with the high determinant density hypothesis.

B.5.3.2 Supporting evidence for these hypotheses

There is evidence for direct recognition by the TCR of MHC polymorphism. Mutations introduced into positions predicted to alter TCR-contacting residues lead to a loss of allorecognition (Ajitkumar et al. 1988). Another observation is that T-cells commonly recognise different class I molecules that share a common antibody epitope (Clayberger et al. 1990). Antibody binding sites are located on the exposed surface of the MHC molecule, implying that the TCR contacts with the MHC framework. In addition, synthetic peptides corresponding to α-helical sequence from the allogeneic MHC molecule can inhibit allorecognition (Parham et
suggesting that these peptides mimic an α-helical portion of the allogeneic MHC molecule and occupy a binding site on the alloreactive TCR that is specific for the foreign MHC structure. In support of the determinant density hypothesis, 'empty' HLA-A2 molecules separated on a column and renatured without peptide still stimulate allore cognition (Elliott et al. 1990). Similar conclusions were drawn by studying mutant lymphoma cell lines, RMA-S or T2, which express limited or no peptides in the context of class I expression, but nevertheless remain sensitive to lysis by some T-cell clones (Ohlen et al. 1990; Heath et al. 1991). However, the level of lysis by these clones can be increased 10-100 fold with the addition of cleaved cytoplasmic proteins. Also, some occupied class I molecules have been shown to occur on these mutant cells, rendering such data ambiguous to interpretation (Henderson et al. 1992).

There is a large body of evidence for peptide dependence in allore cognition. For example, there are many examples of T-cell clones that discriminate between two MHC molecules that differ only at positions in the floor of the peptide binding groove, reflecting a specificity for bound peptide. There is also evidence of tissue specificity in recognition of a murine class II molecule, in which some cells expressing the appropriate class II molecule are unable to stimulate T-cell clones (Lombardi et al. 1989; Marrack et al. 1988). This suggests that the class II is not sufficient to account for allore cognition, a fact that can be explained by tissue differences in expression of proteins that contribute peptides for presentation with class II.

All the data so far, concentrate on the mechanisms of recognition of intact allo-MHC by recipient T-cells. The more physiological means of antigen recognition,
whereby foreign antigen is broken down and presented by recipient class II molecules to host T-cells, has recently come to light as a significant part of the rejection process.

**B.5.4 THE 'INDIREC T' PATHWAY OF ALLORECOGNITION**

**B.5.4.1 Processing of donor MHC molecules**

The basic premise for indirect allorecognition as a mechanism for initiation and/or amplification of allograft rejection is that donor alloantigens are shed from the graft, probably the cell's own MHC, taken up by recipient APC's and presented to T-cells. At least some of the peptides eluted from cell surface class II MHC molecules represent MHC sequences, suggesting that processing of MHC by self APC's may be a physiological event *in vivo* (Chicz et al. 1992a; Chicz et al. 1993). It has also been demonstrated that intact MHC molecules are present in the normal human circulation (van Rood et al. 1970; Charlton et al. 1970) and in renal transplant recipients (SuciuFoca et al. 1991). Processing of these antigens may lead to the activation of T-helper cells, which secrete cytokines and provide the necessary signals for the growth and maturation of effector CTL's and B cells leading to allograft rejection (Parker et al. 1992).

**B.5.4.2 Supporting evidence from animal models**

It is now established that this pathway of donor recognition can contribute to the effector mechanisms of rejection of non-vascularised skin allografts. Fangmann et
pre-immunised rats with peptides derived from donor class I molecules, and demonstrated that skin allografts were rejected in an accelerated fashion (Fangmann et al. 1992a). Dalchau et al. (Dalchau et al. 1992) demonstrated that LEW (RT1\(^1\)) rats primed by immunisation with isolated, denatured (i.e. individual chains) class I molecules derived from DA (RT1.A\(^{wk}\)) rats produce antibodies to the denatured allo-MHC molecules and reject specific skin allografts in an accelerated fashion. These antibodies did not react to the intact MHC molecules. These results suggest that self-restricted T-cell recognition of processed allo-MHC may play a role in allograft rejection. This is emphasised by skin-grafting experiments from MHC class II deficient mice, to donors depleted of either CD4 cells or of CD8 cells (Auchincloss H Jr et al. 1993). They show that CD4 cells are usually required for rapid rejection, meaning that these cells must be recognising donor antigen in the context of recipient class II (as the donor is MHC class II deficient). A further experiment with class II deficient mice as skin graft donors to CD8 depleted recipients shows that the generation of CD8 positive CTL’s requires the help in vivo of CD4 cells, as well as priming with the allogeneic skin graft (Lee et al. 1994). This provides evidence that indirect recognition can provide effective help for CTL induction during graft rejection. The physiological processing of the MHC molecule is important in determining the nature of the immunogenic peptides, as demonstrated by testing the capacity of T-lymphocytes from graft recipients to proliferate in vitro to peptides derived from the donor class I molecule (Fangmann et al. 1992a). Peptides were chosen from different regions of the molecule, and two out of three stimulated CD4 cell proliferation, and a specific antibody response. Benham et al. have recently shown that indirect allorecognition...
of donor antigens can contribute to the effector mechanism of rejection of vascularised organ allografts (Benham et al. 1995) LEW (RT1^d) rats primed for indirect allore cognition of DA (RT1.A^{my}) class I MHC molecules by immunisation with class I derived peptide reject (DAXLEW) F_1 kidney grafts in an accelerated fashion.

B.5.4.3 In vitro evidence for indirect recognition in humans

In humans, the first demonstration of self-restricted T-cell recognition of processed allo-MHC was demonstrated by Dekoster et al. (De Koster et al. 1989) who produced T-cell clones primed by a synthetic peptide derived from the hypervariable domain of the β chain of HLA-DR3. These clones were capable of proliferating to allo-MHC (HLA-DR3) molecules on HLA-DR3/DP3 cells. Proliferation was inhibited by anti-DP monoclonal antibodies, indicating that processed allopeptide was presented by HLA-DP class II molecules. In addition, it has been shown in humans that a large proportion of alloreactive CTL's specific for human class I antigens are self-restricted (BreurVriesendorp et al. 1993), and also that most dendritic cells in human renal transplant biopsies are in fact of recipient origin (Wakabayashi et al. 1993). Thus a definitive demonstration of indirect recognition in clinical transplantation has not yet been reported, although there is strong evidence supporting its presence.
B.5.4.4 The role of indirect recognition in acute or chronic rejection

It has been shown (Liu et al. 1993) by limiting dilution analysis, that the frequency of self-restricted T-cells which recognise processed allo-MHC is approximately 100-fold lower than that of T-cells recognising intact allo-MHC, and suggested that the indirect pathway of recognition may play a minor role in acute, but possibly a major role in chronic allograft rejection. Natural processing of allo-MHC during transplantation may lead to generation of multiple immunodominant peptides. Thus the actual frequency of these T-cells may be underestimated by in vitro assays with a single peptide. The multiplicity of epitopes that may be generated by the processing and presentation of allo-MHC molecules could provide a powerful way of amplifying the alloimmune response to allografts (Shoskes et al. 1994). Further distinction between the role of the indirect pathway in acute and chronic rejection has been proposed by Braun et al. (Braun et al. 1993). They showed that adoptive transfer of a rat CD4 T-cell clone primed by the direct pathway could effect early acute rejection of normal kidney grafts but not of passenger cell depleted kidney grafts (passenger cells being the source of donor APC’s). The implication is that T-cells primed by the direct pathway play a dominant role in acute allograft rejection, but not in chronic allograft rejection, suggesting that T-cells primed by the indirect pathway may play the dominant role in chronic rejection. Depletion of donor class II bearing APC’s occurs naturally after transplantation of vascularised organs (Milton et al. 1984). Donor derived passenger leucocytes migrate out of the graft and are replaced by leucocytes of recipient origin. In this situation, indirect presentation of allopeptides by recipient APC could play a significant role, once the immediate, acute rejection process has
occurred (McKenzie et al. 1984). An additional factor which may play a critical role in the ability of the graft to present antigen once the donor APC’s have migrated out, is the constitutive expression of MHC class II on vascular endothelium in man, but not in rodents. This may go some way towards explaining the relative difficulty in long-term graft acceptance in man, as compared to rodents.

As can be seen, the indirect pathway is implicated in the process of chronic rejection, though as yet there is no categorical evidence experimentally to verify this.

Thus the direct recognition of allo-MHC on the surface of donor cells, and the indirect recognition of processed allo-MHC presented by self APC’s need not be mutually exclusive pathways during rejection, as each is mediated by different sets of T-cell clones. The direct pathway accounts for cytotoxic T-cell function, while the indirect pathway may account for much of Th cell function. Neither the role of indirect allorecognition in chronic rejection, nor evidence for indirect allorecognition in clinical transplant rejection have to date been demonstrated, which were the objectives in our study of human transplant recipients.

B.5.5 CHRONIC REJECTION

B.5.5.1 A continuing problem

The results of solid organ transplantation, in particular kidney transplantation, have steadily improved over the last four decades. This has been secondary to refinements in tissue typing, surgical and peri-operative technique, and more
effective immunosuppression. Concurrently, it has become clear that a significant proportion of grafts fail within the first several months or years after placement, primarily because of progressive and irreversible host immunological attack. The rate of decline has not changed over time, (Clayberger et al.1990), for example less than half of renal allografts from cadaver donors continue to function at six years, despite 80% behaving satisfactorily at one year (Land.1989). The rate of decline of other organ grafts are relatively similar, excepting a lower rate of chronic rejection of liver grafts, perhaps due to a putatively lower immunogenicity of this organ (Starzl et al.1989). This has come to pass in spite of improved immunosuppression. In fact, work in our laboratory has shown that if clonal expansion of T-cells stimulated by the indirect pathway has occurred, this pathway of T-cell help for B-cells is poorly suppressed by cyclosporin (Sawyer et al.1993), suggesting that cyclosporin may be relatively powerless in attenuating late rejection.

B.5.5.2 Risk factors for chronic rejection

Several causes have been implicated, though unproven. The importance of HLA matching remains controversial, although the less steep rate of attrition of kidney grafts from living related donors compared with cadaver donors is the most suggestive data in this respect (Cook.1987). The age of the heart donor has been implicated in chronic rejection, although that of the recipient has not; the incidence of progressive coronary involvement is high even in child recipients (Gao et al.1987; Fricker et al.1987). The influence of immunosuppressive drugs on chronic rejection is doubtful, the effects of cyclosporine nephrotoxicity are difficult to differentiate from chronic rejection. An additional risk factor may be infection of
the recipient with cytomegalovirus early after engraftment (Grattan et al. 1989). In general, the best predictor of chronic rejection in humans, appears to be the number of early, acute rejection episodes.

**B.5.5.3 Pathology of chronic rejection**

The common denominator of the process entails development of obliterative fibrosis of hollow structures within the graft, regardless of whether they are vessels, bronchioles or bile ducts. For example, in the heart coronary artery arteriosclerosis develops, histologically demonstrating cellular proliferation with variable deposition of collagen, extracellular matrix and lipid. Varying degrees (though usually very little) of vascular inflammation may be present. The lesions are composed predominantly of smooth muscle cells, macrophages and T-cells (Hruban et al. 1990). The consistent finding of T-cells and macrophages in graft arteriosclerotic lesions and the demonstration of immunoglobulin and complement deposition in affected vessel walls suggest that these abnormalities occur on an immunological basis (Palmer et al. 1985). In the liver, chronic rejection resembles changes seen in primary biliary cirrhosis and in graft-versus-host disease (Fennel 1981).

In the lung, the term bronchiolitis obliterans was first used to describe the characteristic diffuse obstruction of terminal bronchioles occurring in at least half of the long-surviving (up to 4 years) recipients of heart-lung grafts (Burke et al. 1986; Griffith et al. 1988). No deposits of immunoglobulin or complement have been identified, although expression of MHC class I and an increase in MHC class
II antigens have been found in several biopsies of the pulmonary vasculature and bronchiolar epithelium (Taylor et al. 1989).

B.5.5.4 Experimental models

Many investigators have studied a variety of experimental models to define states of host unresponsiveness towards graft antigens, so-called 'immunological tolerance', and in these hosts some have looked at the late changes occurring within the graft itself. The manipulations to achieve this state mainly involve various chemical immunosuppressive agents, and specific 'blocking' antibodies. For example in cyclosporin treated rats, myocardial fibrosis in long-standing heart allografts has been noted, associated with perivascular cellular infiltration (Baldwin WM et al. 1981). Similar lesions of chronic rejection have also been described in allografts placed in antithymocyte globulin treated rats as well as in MHC compatible non-MHC incompatible hosts (Tilney 1974a; Claesson et al. 1988). Comparable changes have been noted in experimental kidney allografts. A reproducible model of chronic rejection has been described in rat strains differing at 'weak histocompatibility antigens' (White et al. 1969). Seven months after engraftment there are thickened basement membranes, glomerular fibrosis and vessel thickening. Immunoglobulin deposits have not been a consistent finding.

Despite these and other descriptive studies, the origins and progression of the process, particularly in reference to host immunity, are still unclear. The late graft changes seen may represent the invariable result of ongoing subclinical host allore sponsiveness, and seem to mimic chronic rejection in man, although there is no definitive evidence that the processes are one and the same.
B.5.6 THE HOST IMMUNE RESPONSE IN CHRONIC REJECTION

B.5.6.1 The T-cell response

The dramatic histological progression of cellular infiltration coinciding with allograft destruction has stressed the critical role of the cellular immune response in acute rejection. This infiltrate may be less pronounced or even absent in chronically rejecting grafts. Interestingly, few qualitative differences have been found between cell populations infiltrating acutely rejecting, chronically rejecting, or well functioning grafts in unresponsive hosts (Strom et al. 1975; Tilney et al. 1979). The cells, regardless of period of graft function consist of primarily T lymphocytes and macrophages. For example in the bile ducts of chronically rejecting human liver allografts, the ratio of CD8 T-cells to macrophages is considerably higher than in acute rejection, and similarly in the lung of a patient with obliterative bronchiolitis, the CD8 subpopulation was preponderant (McCaughan et al. 1989; Holland et al. 1990). Explaining the presence of the various cell populations in chronic rejection has been difficult.

The lymphocyte responses have been scrutinised in a few experimental models of unresponsiveness (Tilney et al. 1974b; Stuart et al. 1976). No differences in lymphocyte subpopulations were seen in infiltrating cells of acutely rejecting or well functioning rat cardiac grafts at one week, and no significant variations in direct cytotoxicity against donor target cells (Tilney et al. 1977). This finding was confirmed in indefinitely surviving rat kidney allografts (Dallman et al. 1987). The observation that such well functioning grafts contain high numbers of specific cytotoxic T-cells may be partially explained by differences in avidity between
cytotoxic cells, and their targets in the acute or chronic context. In contrast, in human kidney grafts that ultimately failed from chronic rejection, cell populations mounting an anti-donor response were non-T-cells or a mixture of T and non-T-cells, compared to early rejection when T lymphocytes are primarily responsible (Strom et al. 1975). In this context, it is worth emphasising that the long-term non-immunological tissue response is triggered by early immunological injury. Significantly, the role of the macrophage in chronic rejection is unknown.

**B.5.6.2 The humoral response**

The relationship of cytotoxic antibodies to endothelial injury and arteritis in rejection has long been documented, indeed circulating antibodies directed against HLA and other graft antigens are present in immediate or early rejection of organ allografts (Oluwole et al. 1989; Scornik et al. 1989). Humoral activity has also been thought responsible for chronic rejection as immunoglobulin and complement deposits have been seen in areas of intimal thickening (Mohanakumar et al. 1981a). A correlation between the development of graft arteriosclerosis and anti-HLA antibodies has been described (Reemtsma. 1989). In another series, sera collected during the early rejection process was specific against particular HLA loci, whereas sera in late rejection were more multispecific in their activity (Hardy et al. 1979). Antibodies eluted from chronically rejecting allografts show both antidonor specificities of different types as well as non-specific HLA activity (Busch et al. 1976). Antibodies have been described against endothelial cells and kidney cell antigens (Mohanakumar et al. 1981b).
Hence, the specificity of the antibody response in chronic rejection is towards both histocompatibility antigens and also non-MHC tissue components, although the relative importance of this arm of the immune response is unknown.

B.5.6.3 The significance of MHC antigens in chronic rejection

Although acute rejection is triggered primarily by MHC antigens, the chronic process may occur between animals bearing either MHC or non-MHC differences, thus making their importance difficult to judge. Fibrous intimal thickening has been observed in long-standing grafts in both class I compatible rat strains, and in strains differing at both MHC and non-MHC loci (Lurie et al. 1981; Claesson et al. 1988). The influence of MHC antigens is even less clear in some mouse strains. Overall, the early intensity of host alloreactivity (influenced by MHC compatibility as well as other factors, such as immunosuppression) seems to be inversely related to the development of chronic lesions.

Class II antigens are critical in antigen recognition and initiation of host cellular immunity against several organs. Their upregulation occurs on renal tubular epithelium and vascular endothelium during acute cardiac rejection (Milton et al. 1984). Whether this process occurs in chronic rejection is not known, although class II expression has been shown to be normal in human kidneys surviving two years after transplantation (Fuggle et al. 1986). In rat liver allografts, class II differences have been thought to be the most important adverse factors to long survival, whereas class I incompatible grafts could usually survive indefinitely (Tsuchimoto et al. 1985). This apparent significance of class II expression in chronic rejection supports the hypothetical role of indirect allorecognition in this
process. The non-expression of MHC class II on normal rodent vascular endothelium, however, could be a crucial difference in interpreting these experimental models in relation to human vascular endothelium (where class II is constitutively expressed).

Finally, a distinct role for non-MHC antigens expressed on vascular endothelium as a critical antigenic target has been suggested. Immunity toward such non-MHC antigens, with rejection of MHC identical kidneys in pre-sensitised hosts, has been shown in rats (Cheigh et al. 1977), and these antigens may be responsible for the rejection that is occasionally seen in HLA identical kidney graft patients.

**B.5.6.4 T-cell accessory molecules in chronic rejection**

T-cell adhesion molecules are required for the interaction between the TCR and MHC/peptide complex to initiate an immune response. These include lymphocyte function-associated molecule-1 (LFA-1), which binds to intercellular adhesion molecule-1 (ICAM-1), and CD2 which binds to LFA-3 (Martin et al. 1988). Adhesion molecules may be important in the initial events leading to graft rejection. Using ICAM-1 monoclonal antibody renal allograft survival in primates was prolonged by delaying the onset of vascular injury as well as reversing already established rejection episodes (Cosimi et al. 1990). In grafts undergoing chronic rejection, ICAM-1 was less evident in peritubular capillaries, compared with high levels of expression in endothelium and epithelium of acutely rejecting grafts. In human biopsy specimens of allografts undergoing acute rejection, those organs that maintained high levels of ICAM-1 inevitably progressed to chronic rejection (Adams et al. 1989). Thus, although little is known about the role of adhesion
molecules in rejection, it is clear that they are involved in the initiation of acute rejection, and almost certainly contribute to the ongoing cellular damage characteristic of chronic rejection.

As a long-term answer to an irreversible disease process, transplantation has not yet lived up to its potential. Chronic rejection is inexorable and largely uncontrollable, and as yet, immunologically undefined. There is a need for better animal models of the process, as well as a need to marry the experimental findings with the clinical observations.
C.1 ISOLATION AND CHARACTERISATION OF A FRAGMENT OF THE RAT MHC CLASS II TRANSACTIVATOR (CIITA) GENE

C.1.1 INTRODUCTION

Precise regulation of MHC class II gene expression plays a crucial role in the control of the immune response. A major breakthrough in the elucidation of the molecular mechanisms involved in MHC class II regulation has recently come from the study of patients suffering from primary immunodeficiency resulting from regulatory defects in MHC class II expression (also called bare lymphocyte syndrome, BLS). Cell fusion experiments using cell lines derived from BLS patients and a number of experimentally generated MHC class II regulatory mutants demonstrated the existence of several distinct complementation groups. Complementation cloning was first attempted in the cell line RJ2.25, from BLS complementation group A. This led to the isolation of a 4.5kb cDNA (Steimle et al. 1993), transfection of which into cell lines from BLS complementation group A restores wild-type levels of HLA-DR, -DP and -DQ expression, as well as expression of HLA-DMA and -DMB (Kern et al. 1995). Thus CIITA is proposed to act as a non-DNA binding co-activator, as a general controller of MHC class II expression.
The fact that CIITA is essential and highly specific for MHC class II genes makes possible novel strategies designed to achieve immunomodulation via transcriptional intervention. To date, the gene for CIITA has not been isolated in any other species. In transplantation, the rat model has been widely used for experimental studies, and the ability to manipulate the expression of CIITA in this animal would provide a powerful tool for further experimental strategies. One of our specific aims would be to create a transgenic animal, transfected with a construct comprising the rat CIITA gene attached to a promoter specific for expression on vascular endothelial cells (the ICAM-2 promoter). This would test the role of MHC class II expression on vascular endothelial cells during acute and chronic rejection, and thus shed light on the antigen presenting role of class II on vascular endothelium in organ grafts. Clearly, there are numerous other manipulation strategies that may be tried, for example the use of antisense oligonucleotides to attenuate the expression of CIITA and therefore of all MHC class II isotypes. It was for this reason that we decided to attempt to isolate the gene for CIITA in the rat, initially by screening a rat spleen library (which would have a high proportion of class II expressing cells) with the human CIITA cDNA. Following this strategy, cDNA prepared from a rat spleen was used as the template for PCR reactions, using primers designed from the human CIITA sequence.
C.1.2 METHODS

C.1.2.1 SCREENING OF A RAT SPLEEN LIBRARY

C.1.2.2 Materials

LB broth: 10g/l Bacto-tryptone (Difco, Michigan, USA); 5g/l bacto-yeast extract (Difco); 5g/l NaCl (BDH, Poole, UK) pH adjusted to 7.0 with 5N NaOH and autoclaved.

LB broth + 10mM MgSO₄: LB broth was prepared as described above. After autoclaving, it was cooled to 50°C before adding MgSO₄ (BDH) and maltose (Sigma, Poole, UK) for 10mM and 0.2% final concentrations respectively.

LB agarose plates: LB broth was prepared as above. Agarose (15g/l) (NBL, Northumbria, UK) was added prior to autoclaving.

LB agarose + 10mM MgSO₄ plates: LB agarose was prepared as described above. MgSO₄ was added for a 10mM final concentration prior to autoclaving.

LB soft top agarose + 10mM MgSO₄: LB broth was prepared as described. MgSO₄ was added for a 10mM final concentration and agarose (7.2g/l) was added prior to autoclaving.

10X Lambda dilution buffer: 1.0M NaCl; 0.1M MgSO₄.7H₂O; 0.35M Tris-HCl (pH 7.5). Autoclaved.

DNA denaturing solution: 1.5M NaCl; 0.5N NaOH

Neutralising solution: 1.5M NaCl; 0.5M Tris-HCl (pH 8.0)
**Prehybridisation solution:** 6xSSC; 5x Denhardt’s solution (Sigma); 0.5% SDS; 100μg/ml denatured nonhomologous salmon sperm DNA (Sigma)

**TE buffer, pH 8.0:** 10mM Tris.HCl pH 8.0; 1mM EDTA pH 8.0

**TBE, 10 x stock solution:** 0.9M Tris; 0.9M boric acid; 20mM EDTA

**SSC, 20 x stock solution:** 3M NaCl; 0.3M tri-sodium citrate

**Sodium dodecyl sulphate (SDS):** For a 10% solution, 100g of SDS was dissolved in 900ml of distilled water, heated to 68°C. The pH was adjusted to 7.2 with a few drops of concentrated HCl.

**Animals:** Lewis strain rats were obtained from Harlan-Olac (Bicester, Oxon., UK).

**Molecular weight DNA marker:** A 100 base pair ladder was used (Pharmacia Biotech, Uppsala, Sweden) which is prepared by a partial restriction digest of a plasmid. The ladder has uniform gaps of 100 base pairs between fragments, from 100bp to 2000bp and 1μl of a 1μg/μl solution (in TE buffer) was diluted to 10μl in distilled water, and loaded into a well of the agarose gel.

**Library construction:** The cDNA library used was supplied by Clontech (Palo Alto, CA, USA), and was a rat spleen 5’-stretch library. Spleen was chosen as the most likely source of abundant MHC class II and hence CIITA expression. The cDNA library was prepared according to a modified Gubler and Hoffman procedure (Gubler et al.1983), from mRNA which had been completely denatured to release secondary structures. 5’-stretch libraries have larger inserts and therefore have a greater representation of 5’ sequences than do regular libraries.
Higher molecular weight cDNAs are selected and cloned into the λgt11 vector. During preparation, the library was amplified once. The library was supplied in 1X lambda dilution buffer (+0.01% gelatin stabiliser).

C.1.2.3 Electrophoresis of DNA:

a) Reagents: Ethidium Bromide (Sigma Chemicals) was prepared as a stock solution at 10mg/ml in distilled water. This was diluted to give a final concentration of 0.5μg/ml in agarose gels.

Loading buffer (x6 stock solution) was prepared in distilled water, comprising 0.25% bromophenol blue (Sigma chemicals) and 30% glycerol. This was stored at 4°C and diluted 1 in 6 in the DNA to be loaded into each well.

b) Method: Electrophoresis was carried out on horizontal agarose gels using a Pharmacia GNA-100 or GNA-200 gel electrophoresis apparatus. Agarose gels in 1x TBE were prepared by pouring molten agarose with ethidium bromide into a sealed mould containing a well comb. The gel was allowed to set at 4°C. For DNA analysis, 1.0% agarose was used. Samples were loaded after addition of DNA gel loading buffer at a ratio of 5:1 sample to buffer. The gels were run at a power ranging from 80V to 125V for between one to three hours, until the DNA had separated sufficiently, using a Pharmacia electrophoresis power supply type EPS 500/400.

Gels were viewed on a 302nm ultra-violet transilluminator (UVP inc., San Gabriel, USA).
C.1.2.4 **Library titering:**

5µl of the *E.coli* host strain Y1090r from a glycerol stock was streaked onto an LB agar plate containing ampicillin at 50µg/ml. This was incubated at 37°C overnight, and a single isolated colony picked and inoculated into 20ml of LB broth + 0.2% maltose. This was incubated on a shaker (200rpm) at 37°C overnight. The library lysate was diluted to 1:250000 in 1X lambda dilution buffer, and 4 tubes prepared with 0, 2, 5 and 10µl of this library dilution added to 200µl of bacterial culture, and the tubes incubated at 37°C for 15 minutes. 3ml of melted LB soft top agarose + MgSO₄ was added to each of the tubes and mixed, and the contents poured onto 4 separate LB agarose + MgSO₄ plates. The plates were swirled for even spreading, and left to cool, then incubated at 37°C overnight. The number of plaques on each plate was counted to determine the titre (plaque forming units, pfu/ml): pfu/ml = no. of plaques/µl used X dilution factor X 10³µl/ml.

C.1.2.5 **Library screening**

Filter replicas: A single, isolated bacterial colony was picked from the above Y1090r plate, and inoculated into 15ml of LB broth + 10mM MgSO₄ + 0.2% maltose, and incubated overnight as before. Based on the titre obtained above, for each culture plate (a total of six 150mm plates) a sample of diluted phage library was prepared so that the yield would be a maximum of approximately 30000pfu per plate. The dilution was combined with 600µl of bacteria as above, and 7ml of melted LB soft top agarose + 10mM MgSO₄ added to the cell suspension. Each tube was mixed and poured onto a 150mm LB agarose + MgSO₄ plate. The plates
were incubated at 37° C until the plaques were just beginning to make contact with each other.

The plates were chilled at 4° C to allow the LB soft top agarose to harden. Using sterile forceps, numbered nylon membranes (BDH) were placed onto each plate, and the filter marked in 3 asymmetric locations with a sterile needle. After 60 seconds the filter was carefully peeled off, and immersed in DNA denaturing solution for 90 seconds. The filter was then removed and immersed in neutralising solution for 5 minutes, briefly rinsed in 3xSSC and placed on a Whatman 3MM paper (Marathon Laboratory Supplies, London, UK) to dry. A second filter was placed onto the same plate and marked at the same locations. This was lifted off after 3 minutes, and denatured, neutralised and rinsed as above. The DNA on the filters was cross-linked under a UV lamp for 30 seconds.

**Preparation of a DNA probe:** The DNA probe used was a 2kb fragment of the full length human CIITA cDNA (a kind gift of B.Mach, University of Geneva Medical School). The fragment was cut out using Hind III restriction enzyme, purified with the Qiaex II gel extraction kit (Qiagen Ltd., Surrey, UK), and redissolved in distilled water at approximately 50ng/μl.

**Radiolabelling of the probe:** Radioactively labelled copies of the probe were synthesised using DNA polymerase I Klenow fragment and random hexanucleotides as primers. DNA synthesis is primed on denatured template DNA at numerous sites along its length. The components for the reaction were supplied by Amersham International (Amersham, UK).

25ng of the human CIITA DNA probe was denatured by heating to 100° C for two minutes, then chilled on ice. The reaction was set up by adding unlabelled dATP,
dGTP and dTTP (in a buffer solution containing Tris-HCl, pH 7.8, MgCl₂, and 2-mercaptoethanol), the hexanucleotide primer (in an aqueous solution), enzyme solution and ³²P labelled dCTP. The reaction was incubated at 37°C for 30 minutes, and activity of the probe was measured on a pre-calibrated counter (Bioscan QC 2000, Washington DC, USA). The labelled probe was finally denatured as above, and used immediately in the hybridisation reaction.

Hybridisation using the DNA probe: The filters were incubated in prehybridisation solution in glass bottles at 65°C in a rotating oven for 4 hours. The denatured probe was added to the prehybridisation solution in the glass bottles and the filters thus incubated overnight at 65°C in the rotating oven. After hybridisation, the filters were washed in 1X SSC/0.1% SDS at 65°C for 15 minutes, twice, wrapped in cling-film, and left for autoradiography with X-ray film (Sigma Chemicals) on an intensifying screen at -70°C.

C.1.2.6 USE OF THE POLYMERASE CHAIN REACTION (PCR) TO ISOLATE A FRAGMENT OF THE RAT CIITA GENE

C.1.2.7 Removal of rat spleen

A Lewis strain rat (Harlan-Olac, Bicester, Oxon.) was culled in a CO₂ chamber and the chest and abdomen shaved, and cleaned with chlorhexidine solution. Using
sterile instruments, a midline abdominal incision was made, the spleen identified and removed into a petri dish on ice, with sterile normal saline to prevent drying.

C.1.2.8 Preparation of mRNA

mRNA preparation was carried out using the QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). The following solutions were used in conjunction with the kit:

*Extraction buffer* - A buffered aqueous solution containing guanidinium thiocyanate (GTC) and N-lauroyl sarcosine

*High-salt buffer* - 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.5M NaCl

*Low-salt buffer* - 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.1M NaCl

*Elution buffer* - 10mM Tris-HCl (pH 7.5), 1mM EDTA

*Oligo(dT)-Cellulose* - Oligo(dT)-Cellulose at 25mg/ml suspended in a storage buffer containing 0.15% Kathon CG.

All the centrifuge steps were carried out on a desktop microcentrifuge (Microcentaur, MSE, UK) at 13000rpm

0.1g of spleen was weighed out and placed in a 7ml glass pestle and homogeniser, and 0.4ml of extraction buffer added (this contains a high concentration of GTC, ensuring the rapid inactivation of endogenous RNases). The tissue was homogenised until a uniform suspension was obtained, then diluted with 0.8ml of elution buffer (reducing the GTC concentration low enough to allow sufficient hydrogen bonding between poly(A) tracts on the mRNA molecules and oligo(dT) attached to cellulose).
**Isolation of mRNA by binding to Oligo(dT)-Cellulose:** The sample was centrifuged for 1 minute, as was 1ml of the Oligo(dT)-Cellulose to obtain a pellet. 1ml of the homogenate sample was placed on top of the Oligo(dT)-Cellulose pellet and the sample mixed by inverting manually for 3 minutes. The sample was then centrifuged for 10 seconds and the supernatant removed.

**Washing steps:** 1ml of high-salt buffer was added and the Oligo(dT)-Cellulose resuspended. The tube was centrifuged for 10 seconds and the supernatant removed. A total of five washes were done as above with high-salt buffer, followed by two washes with low-salt buffer. The resin was resuspended in 0.3ml of low-salt buffer and the slurry transferred to a MicroSpin column (a polypropylene minicolumn) which was centrifuged for 5 seconds. The effluent was discarded and 0.5ml of low-salt buffer added, followed by another 5 second spin. This wash was repeated twice more.

**Elution step:** The column was placed in a fresh, sterile microcentrifuge tube, and 0.2ml of prewarmed (65°C) elution buffer added to the top of the resin bed. The tube was centrifuged for 5 seconds, and the elution step repeated with a further 0.2ml of elution buffer. The eluate containing the purified mRNA was stored at -35°C.
C.1.2.9 Preparation of first strand cDNA

First-strand cDNA synthesis is catalysed by the Moloney murine leukemia virus (M-MuLV) reverse transcriptase, and involves the full length transcription of mRNA with the addition of dNTP’s and a random hexadeoxynucleotide primer. The components for the reaction are supplied in the First-Strand cDNA Synthesis kit (Pharmacia Biotech) as follows:

**Bulk first-strand cDNA reaction mix** - Cloned murine reverse transcriptase, RNAguard, RNase/DNase-free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer.

**DTT solution** - 200mM aqueous solution

**pd(N)₆ Primer** - Random hexadeoxynucleotides at 0.2μg/μl in aqueous solution

**Procedure**: 20μl of the mRNA solution above was heated in a microcentrifuge tube to 65°C for 10 minutes in a heating block, then chilled on ice. To 11μl of the suspended Bulk First-strand cDNA reaction mix was added 1μl of DTT solution and 1μl of pd(N)₆ primer. The heat-denatured mRNA was added to this mixture, mixed and incubated at 37°C for 1 hour. This yielded a first-strand cDNA reaction product ready for PCR amplification.

C.1.2.10 PCR amplification

PCR amplification using different templates and primers has a wide range of optimal reaction conditions. Two of the most important variables are pH and magnesium concentration in the buffer. There is no way to predict, based on the primer or template sequence, which pH or magnesium concentration will work
best. Therefore we decided to use a series of pH and magnesium combinations, the buffer components being supplied in the HotWax Optistart kit (Invitrogen, San Diego, CA, USA). In this, there are three concentrations of magnesium, 1.5, 2.5 or 3.5mM, and four pH buffers, 8.5, 9.0, 9.5 and 10.0. This gives 12 different permutations of pH and magnesium combinations:

<table>
<thead>
<tr>
<th>pH</th>
<th>[Mg] in mM</th>
</tr>
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<tbody>
<tr>
<td>8.5</td>
<td>1 2 3</td>
</tr>
<tr>
<td>9.0</td>
<td>7 4 8</td>
</tr>
<tr>
<td>9.5</td>
<td>9 5 10</td>
</tr>
<tr>
<td>10.0</td>
<td>11 6 12</td>
</tr>
</tbody>
</table>

The numbers correspond to the lane numbers on the analytical gel, nos. 1-12 being the reactions with spleen cDNA template, and nos. 13-24 are the corresponding negative controls.

In addition, in each reaction, was; 2.5mM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech); 1μl of the above cDNA preparation as template (or 1μl of sterile water in the negative controls); 50ng of each primer; and 1 Unit of Taq polymerase. The reaction volume was made up to 50μl with sterile water. The thermocycler parameters were as follows:

Denaturation 94°C for 2 minutes

cycling steps:

Denaturation 94°C for 1 minute
Annealing  $42^\circ$C for 2 minutes

Extension  $72^\circ$C for 3 minutes

*repeated for 30 cycles*

Final extension  $72^\circ$C for 7 minutes

Once the cycles were complete, 5µl of the reaction could be taken off and loaded (with 1µl of 6x loading buffer) onto a 2% agarose gel, and electrophoresed for analysis.

Primer sequences:

Human CIITA upstream (5’ to 3’) - CAAGTCCCTGAAGGATGTGGA

Human CIITA downstream (3’ to 5’) - ACGTCCATCACCCGGAGGAGGGAC

Actin upstream (5’ to 3’) - GCTGTGGCCATCTCCTGCTCG

Actin downstream (3’ to 5’) - GTATGCTCTGGTGTCGACCAC

C.1.2.11 Cloning of PCR product into a plasmid vector

The TA cloning kit (Invitrogen, San Diego, CA) was used as a cloning strategy for the direct insertion of the PCR product into a plasmid vector. The principle behind this strategy is that *Taq* polymerase has a non-template dependent activity which adds a single deoxyadenosine (A) to the 3’ ends of PCR products. The linearised vector (termed pCR II, supplied with the kit) has single 3’ deoxythymidine (T) residues. This allows the PCR inserts to ligate efficiently with the vector. The pCR II vector incorporates a *lac* promoter and *lacZα* fragment, for bacterial expression of the *lacZα* fragment for α-complementation (blue-white colony screening); a
kanamycin resistance gene; and M13 forward and reverse priming sites for sequencing of the insert.

Reagents (from Invitrogen, unless stated):

**SOC medium**: 2% tryptone (Difco), 0.5% yeast extract (Difco), 10mM NaCl

(DDH), 2.5mM KCl (BDH), 10mM MgCl₂, (Sigma Chemicals)

10mM MgSO₄ (BDH), 20mM glucose (Sigma Chemicals).

β-mercapto-ethanol 0.5M

INVα F' (E.coli) cells

**Procedure:**

**Ligation reaction**: PCR product (calculated from the amount seen on the gel) was added to 50ng of plasmid vector and 4.0 Weiss units of T4 DNA ligase, in a 10μl ligation reaction incubated at 14°C overnight in a water bath.

**Transformation**: Using sterile technique, 2μl of 0.5M was pipetted into a 50μl vial of competent cells and gently mixed. 2μl of the above ligation reaction was added to the cells, mixed gently, and incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds in a 42°C water bath, then placed on ice for 2 minutes. 450μl of SOC medium (at room temperature) was added to the vial, and shaken at 37°C for 1 hour at 225rpm in a rotary shaking incubator. The vial was then placed on ice. 50μl was spread on an LB agar plate, containing 50μg/ml of kanamycin (Sigma Chemicals) and X-gal (NBL). The inverted plates were
incubated overnight in a 37°C incubator, then put at 4°C for 3 hours to allow for proper colour development of the colonies.

C.1.2.12 Purification of plasmid DNA

Plasmid DNA preparation was carried out using a QIAprep kit (Qiagen Inc., Chatsworth, CA, USA), and is based on a modified alkaline lysis method (Birnboim et al. 1979) and on the adsorption of DNA onto silica in the presence of high salt (Vogelstein et al. 1979).

Composition of Buffers:

*Buffer PI (resuspension buffer):* 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml Rnase A

*Buffer P2 (lysis buffer):* 200 mM NaOH, 1% SDS

*Buffer P3 (neutralisation buffer):* 3.0 M potassium acetate (pH 5.5)

*Buffer QBT (equilibration buffer):* 750 mM NaCl, 50 mM MOPS (pH 7.0), 15% ethanol, 0.15% Triton X-100

*Buffer QC (wash buffer):* 1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% ethanol

*Buffer QF (elution buffer):* 1.25 M NaCl, 50 mM Tris-HCl (pH 8.5), 15% ethanol

The principle is the same for both the mini protocol (preparation of up to 20 μg of DNA) and the midi protocol (preparation of up to 100 μg of DNA) from an overnight bacterial culture.
Mini protocol: (All centrifuge steps were on a desktop centrifuge at 13000rpm, unless stated).

1) Preparation of a cleared lysate: 5ml of an overnight culture of *E. coli* in LB medium was centrifuged at 3000g for 10 minutes (Jouan CR4-22, France), and the pellet resuspended in 250μl of buffer P1. 250μl of buffer P2 was added and the tube gently inverted to mix. 350μl of buffer N3 was added and the tube again inverted to mix.

2) Adsorption of DNA onto the QIAprep membrane: The tube was centrifuged for 10 minutes and the supernatant applied onto the QIAprep spin column which was then centrifuged for 60 seconds and the flow-through discarded.

3) Washing and elution of plasmid DNA: 0.5ml of buffer PB was then added to the column followed by a 60 second spin. A further wash as above with 0.75ml of buffer PE followed by an additional 1 minute spin completed the washing steps. The column was then placed in a clean microcentrifuge tube and 50μl of distilled water applied to the centre of the column. This was left to stand for 1 minute, then spun for 1 minute. The eluate containing the plasmid DNA was stored at -20°C until required for further use.

Midi protocol: (All centrifuge steps were done in a Sorvall centrifuge at 20000g and 4°C unless stated).

1) Preparation of a cleared lysate: 25ml of an *E. coli* LB culture was spun at 3000g for 10 minutes (Jouan CR4-22, France), and the bacterial pellet resuspended
in 4ml of buffer P1. 4ml of buffer P2 was added, mixed and incubated at room
temperature for 5 minutes. 4ml of chilled (4°C) buffer P3 was added and incubated
on ice for 15 minutes. The lysate was centrifuged for 30 minutes, the supernatant
removed and spun again for 15 minutes.

2) Adsorption of DNA onto a Qiagen-tip 100 (adsorption column): The
column was equilibrated with 4ml of buffer QBT, then the above supernatant
applied.

3) Washing and elution of plasmid DNA: The column was washed with 20ml of
buffer QC, followed by elution of the DNA with 5ml of buffer QF. The eluted
DNA was precipitated with 0.7 volumes of isopropanol (Sigma) and centrifuged.
The DNA pellet was washed with 2ml of 70% ethanol (Sigma), air dried for 5
minutes, and redissolved in 40μl of distilled water.

C.1.2.13 Ethanol precipitation

This was carried out on samples that required purification from protein
contamination and/or required concentration into a smaller volume of diluent.
The following were added to the sample to be precipitated: 1/10th of the sample
volume of 3.0M sodium acetate, pH 5.2 and 2.5 combined (sample + sodium
acetate) volumes of ethanol at -20°C. The mixture was left at -20°C overnight,
allowed to thaw, and microcentrifuged for 10 minutes. The supernatant was
removed, and the sample washed with 70% ethanol at -20°C, and centrifuged as
above. After careful removal of the supernatant, the pellet was dried under vacuum (DNA Speedvac 110, Savant, Farmingham, NY) for 5 minutes, and dissolved in a suitable volume of distilled water.

C.1.2.14 Sequencing reactions

An ‘AutoRead sequencing kit’ (Pharmacia Biotech) was used for the sequencing reactions and involves the use of the bacteriophage T7 DNA polymerase. The system employs a non-radiochemical approach to sequencing, in which a primer labelled with fluorescein at its 5'-terminus is annealed to the double-stranded template. Using standard dideoxy sequencing methods (wherein each termination mix contains a mixture of three dNTPs, with the fourth being a dideoxynucleoside NTP, ddNTP), the fluorescent primer is extended by T7 DNA polymerase in four separate dideoxy reactions (A,C,G and T), creating four separate populations of fluorescently labelled chain-terminated fragments. Each termination mix stops elongation of the polynucleotide at the appropriate sequence residue due to the lack of a 3'-hydroxyl group. The reactions are then loaded into four adjacent lanes on a sequencing gel and electrophoresed.

Components:

**A mix:** 5μM ddATP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP.

**C mix:** 5μM ddCTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP.

**G mix:** 5μM ddGTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP.

**T mix:** 5μM ddTTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP.

All mixes also contain 50mM NaCl and 40mM Tris-HCl (pH 7.6).
**T7 DNA polymerase:** 8 units/μl in 25mM Tris-HCl (pH 7.5), 0.25M NaCl, 5mM DTT, 50% glycerol.

**M13 Universal primer:** 5’-fluorescein-d-3’ in aqueous solution, 1.5 μM.

**M13 Reverse primer:** 5’-fluorescein-d-3’ in aqueous solution, 1.5 μM

**Annealing buffer:** 1M Tris-HCl (pH 7.6) and 100mM MgCl₂.

**Extension buffer:** 304mM citric acid, 324mM DTT and 40mM MnCl₂ (pH 7.5).

**Stop solution:** 100% deionised formamide and dextran blue 2000 (5mg/ml).

**Procedure:**

1) **Annealing of primer to double-stranded template:** To 10μl of template DNA (at approx. 1μg/μl) was added 2μl of fluorescent primer (universal or reverse), and 1.5μl of 1M NaOH. The mix was incubated at 65°C for 5 minutes, transferred to 37°C and 1.5μl of 1M HCl and 2μl of annealing buffer added. This was incubated for 10 minutes, then placed at room temperature for 10 minutes. 1μl of extension buffer and 3.5μl of DMSO were then added.

2) **Sequencing reactions:** 4 Units of T7 DNA polymerase were added to the annealing reaction, and 5.4μl of this mixture added to 3μl of each of the (A,C,T and G) sequencing mixes. These were incubated for 5 minutes at 37°C then 5μl of stop solution added to each mix. The reactions were heat denatured at 90°C for 3 minutes, then quenched on ice. 10μl was loaded into the appropriate wells of a sequencing gel.
C.1.2.15 DNA sequencing apparatus

The ALF™ DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) was the automated sequencer used. As the DNA fragments in each lane migrate through the gel, they pass a fixed laser beam, which excites the fluorescently labelled molecules, and the light emitted is detected by the photodetectors. The four signals for each clone are converted into serial digital data which is then stored automatically for subsequent sequence determination. For each base in the sequence, an intensity peak is present at a point of time relative to the position of the base in the sequence. The order of the peaks thus corresponds to the base sequence of the clone.

The glass plate and thermoplate of the gel cassette were washed with deionised water and 100% ethanol, as detailed in the ALF protocol. The top 5cm of the glass plate was treated with Bind-Silane solution (Pharmacia), and the gel cassette assembled.

Sequencing gel:

A 50% liquid concentrate of an acrylamide monomer was used, called Long Ranger (HydroLink, Malvern, PA, USA).

This was made up as follows in 50mls deionised water:

- 21g Urea (Pharmacia)
- 6ml 10xTBE
- 5ml Long Ranger concentrate
- 0.025ml TEMED (Sigma Chemicals)
- 0.25ml 10% ammonium persulfate (Sigma Chemicals)
The acrylamide gel was prepared, cast on the gel cassette, and left to polymerise for 90 minutes. The cassette was then loaded into the sequencer and 0.6xTBE running buffer added to the reservoirs. The samples were loaded onto the gel, 10μl per lane, and the following electrophoresis run conditions used:

- Operating voltage: 1900V
- Operating current: 65mA
- Operating power: 35W
- Laser power: 4mW
- Water temperature: 47°C
- Sampling time: 1.25 seconds

The completed sequence was processed by the ALF manager programme, version 2.6.

C.1.3 RESULTS

C.1.3.1 Library titering

The library titre was calculated to be approximately 5 X 10⁹ plaque forming units/ml. This figure was used to calculate the dilution and amount of the library, to combine with the appropriate quantity of bacteria per culture plate. All six plates yielded approximately the same number of plaques after overnight growth, and a sample count on one of the plates estimated 6-10000 plaques per plate.
After the plaques had been lifted from the plates, and labelled with the probe, the filters were washed until a Geiger counter indicated that the count per minute was between 10000 and 20000.

C.1.3.2 Library screening

After 24 hours of exposure of the X-ray film to the radiolabelled filters, there were occasional strong, sharp signals on the film which were not duplicated on the appropriate replica filter, and therefore taken to be spurious electrical discharge. Weaker signals were present, so a 72 hour exposure was done, which made these signals more apparent, but these were not duplicated and were not indicative of specific hybridisation. The moist filters were stripped of radioactive probe (by immersion in boiling 0.5% SDS) and re-probed as above, this time with less stringent washing of the filter (2X SSC/0.1% SDS at 65°C for 15 minutes, twice). This had the effect of increasing the background signals, but again indicated no specific hybridisation. This led us on to the next strategy for isolation of the rat CIITA gene.

C.1.3.3 PCR of rat cDNA using human primers

A spleen was removed from a Lewis rat and mRNA prepared from 0.1g of tissue in a final volume of 400μl. From this preparation, 20μl was taken, and 33μl of cDNA prepared as described above. This cDNA was then used as the template in an optimised PCR reaction, with mouse cDNA (from a BALB/c strain mouse spleen, isolated in the same manner as above) or no cDNA in the control reactions. The primer pairs used were either PCIITA1 and PCIITA2 (derived from the human
CIITA sequence), or actin primers as the positive control. The PCR reactions were run out on a 2% agarose gel, for 30 minutes at 125V. All the reactions with rat cDNA gave a number of positive bands in each lane, with the strongest band in all the lanes being of the appropriate size for the primers used (figure 1a). All the negative control reactions (no cDNA or mouse cDNA used) were blank (figure 1b), except for the primer-dimer band seen, wherein the primers do not bind to a template. The actin positive control also gave an appropriately sized band, as well as a weaker, slightly larger band which is spurious, and likely to have been a consequence of the low annealing temperature used in the reaction. The human CIITA primers had already been seen to give the appropriately sized product when used with human CIITA cDNA as the template in a PCR reaction (data not shown).

C.1.3.4 Cloning of the PCR product into a plasmid vector

The positive band described above, derived from rat cDNA, was indicative that this was the equivalent segment of rat CIITA to the human CIITA fragment. To confirm this, it was necessary to sequence the fragment obtained. To sequence this fragment would involve cloning the PCR product into a suitable plasmid vector, which could then be transformed into a bacterial strain, which could be grown in culture in order to obtain enough purified DNA for sequencing purposes.

1μl (out of a total volume of 50μl) of the cleanest PCR product, namely lane 11, was used in a 15μl reaction incorporating 2μl of PCR vector from the TA cloning system (Invitrogen). This would give an estimated molar ratio of 1:1 of vector to PCR insert. A control (self-ligation) reaction was also set up as above but with no
PCR product. The ligation reactions were left overnight at 12°C. The remainder of
the PCR product was stored at -35°C.

C.1.3.5 Transformation of ligated PCR product into INVαF' cells

1μl of each ligation reaction was used in the transformation procedure described,
and the cells plated onto LB Agar plates with X-gal for colony selection. A total of
9 white colonies (indicating recombination) were seen on the PCR product plates,
with just 1 blue colony (indicating self-ligation of the vector, and hence expression
of the lacZ gene). On the control plate there were two blue colonies and no white
colonies, indicating that the vector was stable.

C.1.3.6 Isolation of DNA from the transformed cells

All the white colonies were picked from the plates with sterile loops, innoculated
into 5mls of LB broth with kanamycin (Sigma Chemicals) at 50μg/ml, and grown
overnight at 37°C in a gyratory shaker-incubator at 225rpm. 850μl of each
bacterial growth was then added to 150μl of glycerol and stored at -35°C in
microcentrifuge tubes. The rest of the growth was then spun down and plasmid
DNA isolated (QIAprep mini kit) as described.

5μl out of 45μl of each DNA sample was then digested with the restriction enzyme
EcoRI (Promega, Southampton, UK) (5μl plasmid DNA, 1μl 10X buffer, 12 Units
enzyme in a 10μl reaction) in a 37°C water bath for 2 hours. The insert site in the
plasmid is flanked by EcoRI restriction sites, so this enzyme would cut out any
insert. 2μl of 6X loading buffer was added to 10μl of the digestes and run out on a
2% agarose gel, at 125V for 2 hours. The resulting gel showed that in 4 of the
picked colonies there is a distinct band of the predicted size (figure 1c). In 1 colony there were 2 bands, in another there was a smaller than expected band, and in three colonies there were no detectable inserts.

Lanes 3 and 5 as seen on this gel were chosen for further analysis. The equivalent glycerol stock cultures were taken and, using a sterile loop, a small amount inoculated into 25mls of LB medium with kanamycin at 50μg/ml. This was shaken overnight at 37°C as above, and the resultant bacterial culture used to prepare plasmid DNA as described (QIAprep midi kit), in sufficient quantity for sequencing reactions, in a total volume of 20μl. The amount and purity of the DNA obtained was checked on a spectrophotometer (Ultrospec III, Pharmacia Biotech) using a 1/200 dilution of the DNA in a 1ml UV cuvette. This indicated a total yield of 35μg of DNA from colony 3, with a 260nm/280nm ratio of 1.6, and 19μg of DNA from colony 5, with a 260nm/280nm ratio of 1.8.
Figure 1: PCR products using optimisation protocol, with 12 different buffer combinations. (a) lanes 1-12 = human CIITA primers on rat cDNA (b) lanes 13-24= negative controls (no cDNA), lane 25= actin control, lane 26= human CIITA primers on mouse cDNA. Buffer combination ‘8’ was used in reactions 25 and 26. M = 100bp marker lane
Figure 1c: PCR products cut out from plasmid vector by Eco RI (lanes 1-9) (M = 100bp marker lane). The insert appears at approximately 400 base pairs.
C.1.3.7 **Sequencing of the plasmid DNA**

1μg of DNA is required per kilobase to be sequenced, in each sequencing reaction. The total length of plasmid plus insert is 4.3kb, so a minimum of 5μg of plasmid DNA per reaction was required. In fact an estimated 10μg of DNA was used in each reaction to maximise the number of bases read. The sequencing procedure was carried out as described, on DNA derived from colony 3, with 2 reactions, using both forward and reverse primers, in order to sequence the insert from both ends.

The resulting sequencing run read 445 bases in the forward direction, and 626 bases in the reverse direction, with high quality peaks making reading of individual bases clear to the end of the run (figure 2). The PCR primer sequence started at approximately 60 bases into the sequencing run, followed by a 434 base insert, followed by the complementary primer. The sequence of the insert was confirmed by both forward and reverse readings, and was compared to the human CIITA sequence to confirm that it was sufficiently homologous that it could be established to be a fragment of the rat CIITA gene.
Figure 2: Sample section of DNA sequencing run. DNA derived from colony 3, using reverse primers, showing a portion of the rat CIITA gene isolated.
Figure 3a: Nucleotide sequence of rat CIITA PCR fragment, compared with homologous human sequence. ‘Non-silent’ base substitutions in the rat sequence are indicated in bold type.
Figure 3b: Predicted amino acid sequence of the rat CIITA fragment, using the one letter code. Substitutions from the corresponding human protein sequence are shown.
C.1.3.8 Analysis of the rat CIITA gene fragment

The product sequenced was 433 base pairs in length (figure 3), with the equivalent position on the human cDNA sequence of bases being base 2899 to 3333, of a total 4.5kb gene. There were 92 base substitutions, giving an overall sequence homology between rat and human of 78.6%. Of these substitutions, 57 were 'silent', with no consequent amino acid substitution, and 35 resulted in an amino acid substitution in the protein sequence. The amino acid sequence had 29 substitutions (out of 143 translated amino acids) compared to the human protein sequence, giving an overall protein homology of 79.7%.

C.1.4 DISCUSSION

CIITA is an overall controller of MHC class II expression by its action in binding to proteins that bind to the promoter region of the class II gene. Its action is specific, and exerts control on all MHC class II isotypes. The gene for CIITA was initially identified in patients with MHC class II deficiency, and has not to date been identified in any other species.

Because the CIITA gene is so specific in controlling the expression of all class II isotypes, its cloning in the rat would serve as a very useful tool in the manipulation of MHC class II in the experimental transplantation model. For example, class II molecules are not expressed constitutively on the vascular endothelium of rodents, in contrast to human vascular endothelium. The vascular endothelium is a permanent constituent of an organ graft that may possess antigen presentation capacity via its expression of class II, and hence be a significant component in the
chronic rejection process seen in clinical organ transplantation, but not seen in experimental rodent transplants. The rat CIITA gene could thus be used in a transgenic experiment, in which a promoter specific for a molecule expressed constitutively and exclusively on vascular endothelium (e.g. the ICAM-2 promoter, the cDNA of which we have obtained as a kind gift of T. Springer, Boston, USA) would be attached to the CIITA gene, as the transgene construct. In this way it would be possible to express MHC class II on the vascular endothelium of long-term rat allografts, as is the situation in humans, and test this hypothesis.

Our search for the rat CIITA gene was initially based on the possibility of sufficient homology between the human and rat CIITA sequences, to allow hybridisation under the right conditions of a portion of the human CIITA cDNA to the equivalent sequence in a rat genomic library. This proved not to be the case, and a number of possible explanations are discussed. Firstly, the titre of plaques obtained gave a theoretical yield of 30000 pfu per plate, so that screening six plates should screen 180000 plaques. In reality, a sample count of plaques on one plate, indicated that there were approximately 6-10000 pfu per plate, meaning that a maximum of 60000 plaques were screened. For an infrequently occurring gene in a genomic library (as CIITA is likely to be), it would be ideal to screen 100-150000 plaques. Thus, we may simply have been unlucky in that the cDNA for CIITA was not present in any of the plaques screened. Secondly, it may be that the fragment of gene used as a probe was sufficiently non-homologous as to not bind to rat CIITA even under the non-stringent washing conditions described. As mentioned, the gene for CIITA has not been identified in any other species to date. The RFX family of genes, however, has been identified in a wide variety of eukaryotic
species, including the mouse, with between 70.9% and 100% homology to the human sequences (Emery et al. 1996). The CIITA PCR fragment isolated demonstrated 78.6% sequence homology with the human sequence, which is adequate for cross-species hybridisation under non-stringent conditions (as we used). In addition, this fragment is present within the radiolabelled probe used for library screening. Thus, theoretically, it should be possible to label the rat CIITA gene with human CIITA probe, if this level of homology persists throughout the probe sequence. However, instead of persevering with a potentially large number of experiments using many different stringency and hybridisation conditions, we chose to use the PCR approach, to obtain a far more specific probe. Finally, it is conceivable that the gene for CIITA in the rat has been silenced during the course of evolution, and so does not appear in a cDNA library. This possibility is excluded by our success with PCR on cDNA samples.

The PCR technique described was eventually successful after trying a number of different primers designed from the human sequence. The annealing temperature was also important, as the use of higher (and therefore more stringent) temperatures, yielded no product.

The fragment obtained can now be used for further probing of the rat library, or for design of antisense oligonucleotides. We have obtained experience in the use of antisense technology, with this in mind, and detail the findings in the next data chapter. Another strategy is to design rat specific primers from this sequence to use with complementary human primers further up or down the sequence, or primers from around the insertion point of the phage vector sequence, in an 'extended' PCR experiment, in order to obtain longer fragments of the rat gene. Our initial
attempts at this latter approach have not been successful, although there are a number of further combinations to investigate. The eventual cloning of the full length gene by these strategies is facilitated by the isolation and characterisation of the rat CIITA PCR fragment, and will provide the means for the setting up of more sophisticated experimental models.
C.2 THE USE OF ANTISENSE OLIGONUCLEOTIDES TO INHIBIT T-CELL PROLIFERATION IN VIVO

C.2.1 INTRODUCTION

Antisense oligodeoxynucleotides (oligos) hold great promise as agents for specific manipulation of gene expression, by blocking the expression of specific genes within cells. This inhibition relies on the ability of an antisense oligo to bind a complementary messenger RNA sequence and thereby prevent translation of the mRNA, either by physical blocking of translation or by Rnase H cleavage of the mRNA (figure 1). Antisense oligo effects have been reported in mammalian cells in numerous tissue culture experiments, and more recently in several in vivo studies (e.g. Osen Sand et al. 1993). Clinical trials have been initiated to evaluate the therapeutic potential of antisense oligos in several human diseases, including acute myelogenous leukemia and cytomegalovirus infection (Bayever et al. 1993).

There are, however, many biological effects of antisense oligos that cannot be attributed to antisense mechanisms alone (Stein et al. 1993). Such effects may limit the conclusions that can be drawn from antisense experiments, and may also compromise the usefulness of antisense oligos as therapeutic agents. Recent advances, however, have led to the development of more potent and specific antisense methods.
Non-specific effects of antisense oligonucleotides

Despite numerous reports of apparent antisense inhibition of gene expression in cultured cells, only in a few cases has specific inhibition been rigorously demonstrated. In many studies, specificity has been inferred from the biological effects of antisense as compared to control oligos, without measuring levels of target RNA or proteins to evaluate specificity. Without such controls, however, it is difficult to exclude the possibility that an observed effect may be due to actions other than blockage of the intended target RNA. Unintended side-effects could potentially occur through a number of possible mechanisms. Antisense oligos have been shown in *Xenopus* to cause cleavage of imperfectly matched target sites (Woolf et al. 1992), thereby inhibiting the expression of genes other than the intended target. Sequence specific and non-specific binding of oligos to small molecules (Ellington et al. 1990) and proteins (Bock et al. 1992) have also been described. Non-specific cellular activation of the SP1 transcription factor by phosphorothioate oligonucleotides has been reported (Perez et al. 1994). Antisense oligos can also inhibit viral infection by non-antisense mechanisms that are not fully understood, but which may include interference with absorption, penetration or uncoating of virus (e.g. Yakubov et al. 1993). Finally, nucleosides and nucleotides, the degradation products of antisense oligos, can affect cell proliferation and differentiation (Kamano et al. 1992). Because oligos are degraded both intra- and extracellularly, these breakdown products may account for some of their observed effects, especially at higher concentrations.
Figure 10: Mechanism of action of antisense oligos

Target Gene

antisense DNA strand

sense DNA strand

TRANSCRIPTION

Messenger (sense) RNA

Antisense Oligo

PROTEIN SYNTHESIS BLOCKED

or

RNase H hydrolysis
Controls for measuring antisense effects

It is becoming more and more clear, that demonstrations of antisense gene inhibition need to be meticulously isolated from the non-specific biological effects of antisense oligos. Currently, ideal experimental controls include the following:

Direct measurement of the target RNA or protein levels as compared to an internal control RNA or protein. RNase H hydrolyses the RNA strand of a RNA-DNA duplex and is likely to be responsible for the antisense effects of 2'-deoxy-oligonucleotides. Thus, the degree of degradation of the target RNA may be a good assay for gene inhibition. Direct measurements of effects on protein levels may be more problematical, because if protein turnover is slow, some time is needed before a reduced rate of synthesis is detected. Another problem arises in cases where the target protein regulates synthesis of other proteins (as, for instance, with cell-cycle proteins). In such cases, selection of appropriate controls is difficult, and the best control may be to use several active antisense oligos targeted to the same RNA as a demonstration of specificity.

Mismatched control oligonucleotides are a crucial element in the design of an antisense experiment. Many studies have used a sense oligo complementary to the antisense sequence, whereas others have used oligos of the same length as the antisense oligo, but composed of a random mixture of all four nucleotides. Because oligonucleotides may be degraded in culture, however, the best control sequences are those that contain the same base composition as the antisense sequence. Ideally the control should differ from the antisense sequence no more than is necessary to prevent specific hybridisation.
Characteristics of chosen oligonucleotides

Oligonucleotides containing phosphodiester linkages are rapidly degraded in most cells, and in tissue fluids, with a typical half-life of around 20 minutes. Oligos can be made resistant to nucleases by introducing modifications such as phosphorothioate linkages (figure 2), which is essential for high activity (Fisher et al. 1993).

Careful choice of target sequence is important, but the ultimate choice is still largely empirical. Many investigators have chosen to target the initiation of translation codon of the mRNA on the assumption that the region is important and accessible. Recent studies indicate that most regions of the mRNA are in fact accessible to oligos, except for those with strong secondary structure, and the chemistry of the oligonucleotide modification appears to play a significant role in the relative efficacy of different sites (Wagner et al. 1993). The reasons for these effects are not well understood.

Further refinements

The importance of cell permeability has become more apparent recently, with most studies showing permeabilisation to be absolutely necessary. The potency of antisense oligos can be greatly enhanced by the use of cationic liposomes which appear to augment the exit of oligos from the endocytic vesicles. For example 100nM of phosphorothioated oligo is sufficient to reduce ICAM-1 protein levels in endothelial cells by 90%, whereas no effect is seen in the absence of liposomes (Bennett et al. 1992). Similarly, in the presence of cationic liposomes, a concentration of 200nM of oligo is sufficient to produce a 50-80% decrease in
RNA and protein levels for the (human procollagen) target, whereas no effect was seen in the absence of the liposome, even at a concentration of 25μM of oligo (Colige et al. 1993).

The use of antisense oligonucleotides in the transplantation setting

In transplantation, the processing of alloantigen, by whatever means, leads to effector T-cell activation which is dependent on cytokine signalling pathways. The use of monoclonal antibodies (mAb) to inhibit specific signalling molecules (e.g. Lenschow et al. 1992) has proved an effective means to prolong graft survival and induce tolerance in the experimental model. The use of mAbs, however, is inhibited by their long-term limitations of inducing a host antibody response to themselves. This is more likely to be an obstacle in human transplantation, where long-term immunosuppression remains the norm. This has led us to explore the possibility of using antisense technology as a viable alternative in the specific inhibition of cytokines involved in the T-cell activation pathway.

IL2, principally a product of CD4 T-cells, stimulates proliferation of CD4 cells in an autocrine fashion, and leads to paracrine activation of CD8 lymphocytes. IL-2 exerts its function through a membrane receptor (IL-2R), and the IL-2/receptor complexes are internalised via receptor mediated endocytosis. In addition, it has been shown that an altered regulation of the IL-2 pathway results in tolerance in transplanted animals, with reversal of the tolerogenic effect by administration of recombinant IL-2 at the time of transplantation (Dallman et al. 1991b). Also, CD28 deficient mice have been shown to have impaired IL-2 production from lectin
stimulated T-cells (Shahinian et al. 1993). It is reasonable to suppose that tolerance may result from an allograft response conducted in the absence of IL-2 or B7 expression.

To date, most studies using antisense oligonucleotides have focused on inhibition of expression of viral genes and oncogenes. To assess the capability of these agents to modulate cytokine expression, and thereby to study the effect of inhibiting specific cytokines on cellular proliferation, we designed species-specific oligodeoxynucleotides antisense to the mRNA sequence for mouse IL-2, IL-2R and B7.

The experiments that we performed were designed to test the effect of these antisense oligos on an easily reproducible in vitro system of cytokine dependent T-cell proliferation. We used both the mixed lymphocyte culture (MLC) and also concanavalin A stimulated T-lymphocytes as the model systems in which we could test the effects of the oligos, by co-incubation, in the culture.

Antisense technology has been rapidly evolving over the last few years, and the importance of cell permeabilisation techniques has been one of the most recent refinements. The initial set of experiments were done before this particular era, in other words without the use of a permeabilising agent. The data are instructive in the way that antisense effects are often highly promising, but that the rigorous demonstration of controls is more complex.
Figure 2: General molecular structure of normal (X=O) and phosphorothioate (X=S) oligomers. B is adenine, guanine, cytosine or thymine.

Structure of oligodeoxynucleotides
C.2.2 MATERIALS AND METHODS

C.2.2.1 Oligonucleotide design and synthesis

Oligonucleotides were synthesised complementary to unique species-specific coding regions of mRNA for the target molecule (table 1). All were phosphorothioate end-protected, and synthesised via phosphoramidite chemistry by sulphurisation with tetraethylthiuram disulfide in acetonitrile (the oligos were synthesised by Kings College, London SE5, UK). The oligos, once received, were purified by ethanol precipitation, and the concentration determined by UV absorption at 260nm (Ultrospec III, Pharmacia, Sweden). They were then diluted to a concentration of 10μM in RPMI 1640 medium (Flow Labs., Irvine, Scotland) (experiments 1-4) or serum-free medium (Opti-MEM, Gibco, Paisley, Scotland) (experiments 5-7), aliquoted and stored at -20°C until needed.
Table 1: Antisense oligonucleotides used

<table>
<thead>
<tr>
<th>TARGET MOLECULE</th>
<th>OLIGONUCLEOTIDE SEQUENCE (5' TO 3')</th>
<th>REGION OF GENE TARGETED</th>
<th>NOMENCLATURE USED</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>ACAATTGCAAGCCATAGCTT</td>
<td>covering initiation codon</td>
<td>B7as</td>
</tr>
<tr>
<td>IL-2R</td>
<td>CAAGCGTGGCTCCATCTTCC</td>
<td>covering initiation codon</td>
<td>IL-2Ras</td>
</tr>
<tr>
<td>IL-2</td>
<td>CTGCATGCTGTACATGCCTG</td>
<td>covering initiation codon</td>
<td>IL-2as/1</td>
</tr>
<tr>
<td>IL-2</td>
<td>CACCCCTGCTAATCCTCCT</td>
<td>24bp upstream of initiation codon</td>
<td>IL-2as/2</td>
</tr>
<tr>
<td>IL-2</td>
<td>AGCTCGCATCCTGTGTC</td>
<td>10bp downstream of initiation codon</td>
<td>IL-2as/3</td>
</tr>
<tr>
<td>CONTROL</td>
<td>GTCCGTACATGTACGTACGT</td>
<td>reverse orientation (3’ to 5’) of IL-2as/1</td>
<td>IL-2ns</td>
</tr>
<tr>
<td>CONTROL</td>
<td>TACATACGTGGCTCCGCTCGT</td>
<td>same bases as IL-2as/1 in a random order</td>
<td>IL-2nsj</td>
</tr>
<tr>
<td>CONTROL</td>
<td>TTCGATACCGAACCCTCCTAACA</td>
<td>reverse orientation (3’ to 5’) of B7as</td>
<td>B7ns</td>
</tr>
<tr>
<td>RAT IL-2 (CONTROL)</td>
<td>TTCGTACATGTACGTACGTCG</td>
<td>covering initiation codon</td>
<td>RIL-2</td>
</tr>
</tbody>
</table>
C.2.2.2 **Animals**

Mouse strains used were BALB/c (female), CBA/Ca (male), C57BL/10ScSn (male) and C3H/He (male) (Harlan Olac UK Ltd., Bicester, Oxon). All were greater than 6 weeks old.

C.2.2.3 **Cell cultures (protocol for experiments without liposome)**

Lymphocytes from mouse cervical and mesenteric lymph nodes, and from spleen were removed in a sterile manner detailed in section C.5.2. The tissue was dispersed into a single cell suspension using sterile scissors and a sterile sieve (Marathon, London, UK), washed twice in Hepes buffered RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 1% foetal calf serum (Gibco), 2mM glutamine, $5 \times 10^{-5}$ M 2-mercapto-ethanol, 100 units/ml penicillin and 100µg/ml streptomycin (all supplied from ICN, Bucks., UK). In mixed lymphocyte culture experiments, at this stage the stimulator spleen cells (in a total volume of 10 ml, at $4 \times 10^6$ cells/ml) were irradiated with 2000 Rads (Gammacell 1000, Nordion, Ontario, Canada). All cells were then counted, pelleted, and re-suspended at a concentration of $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% foetal calf serum and glutamine, 2-mercapto-ethanol, penicillin and streptomycin as above.

$2 \times 10^5$ responder cells were incubated in triplicate in round-bottomed, 96 well tissue culture plates (Sterilin, Feltham, England) in 200µl volumes of the above culture medium. Cultures were stimulated with $2 \times 10^5$ stimulator cells, or with Concanavalin A (Sigma Chemicals, Poole, Dorset, UK) at 10µg/ml. Oligonucleotide (dissolved in RPMI 1640 or Optimem medium, see above) was
added at the appropriate concentration before incubation (see figures 1-6 for specific concentration for each experiment). Incubations were at 37°C in 5% CO₂ in air. 24 hours before harvesting, 1μCi of ³H thymidine (Amersham International, Bucks., UK) in 20μl of culture medium was added to each well. Cell-bound radioactivity was measured by placing dried filters (ICN, Bucks., UK) in Optiscint Hisafe scintillation fluid (LKB, Milton Keynes U.K.) and counting in a LKB Rackbeta II liquid scintillation counter (LKB, Bromma, Sweden).

C.2.2.4 Cell cultures (protocol for experiments with liposome)

The first step involved binding of the oligonucleotide to the liposome. Lipofectamine (Gibco, Paisley, Scotland) was diluted to a concentration of 40μg/ml in serum-free medium (Opti-Mem, supplemented with 5x10⁻³ M 2-mercapto-ethanol, 100 units/ml penicillin and 100μg/ml streptomycin). To this was added oligo at a concentration of 4μM, and the solution incubated at room temperature for 50 minutes. Lymphocytes were obtained from lymph nodes as before, dispersed into a single cell suspension, and washed twice in RPMI 1640 supplemented with 2mM glutamine. The cells were counted, and resuspended in the above oligo/liposome preparation at a concentration of 1.5x10⁶ cells/ml, with the final concentration of oligo being 1μM and that of lipofectamine being 10μg/ml. The cells were plated into a 24 well flat-bottomed plate (Sterilin, Feltham, England) using 2mls/well, and incubated for 4 hours at 37°C in 5% CO₂ in air, with gentle resuspension every 30 minutes. After this incubation, the cells were transferred to 15ml tubes, washed twice in RPMI 1640 + glutamine, and resuspended in RPMI 1640 supplemented with 10% foetal calf serum and
glutamine, 2-mercapto-ethanol, penicillin and streptomycin as above, with added
con A at 5µg/ml. This culture was plated into a 24 well plate, and incubated at
37°C in 5% CO₂ in air for the required number of days, at which point the culture
supernatants were removed for study of IL-2 activity (see below).
For measurement of cell proliferation, a proportion of the cells prepared were
plated out in triplicate in 96 well plates, at 200µl/well, at the same concentrations,
instead of the 24 well plates used above. The thymidine incorporation and
harvesting were then exactly as detailed above.

C.2.2.5 Use of IL-2 dependent cells to assay IL-2 activity of culture
supernatants
CTLL cells (ECACC, Porton Down, Salisbury, UK) were grown in culture to
confluence in RPMI 1640 supplemented with 10% foetal calf serum and
glutamine, penicillin and streptomycin as above, with the addition of recombinant
IL-2 (rIL-2) (Genzyme, Cambridge MA, USA) to maintain the culture. These cells
were taken, washed and resuspended in the above medium at 2x10⁵ cells/ml. They
were plated into 96 well flat-bottomed plates (Sterilin, Feltham, England) at
50µl/well, using two rows for each supernatant tested. To the first column (out of
12) 50µl of undiluted culture supernatant from above, or 50µl of rIL-2 at 100U/ml,
was added. This was mixed, and 50µl removed and added to the next column
along. The process was repeated for all 12 columns on the plate, to give serial
dilutions of the supernatants being mixed with the CTLL cells.
The cultures were incubated at 37°C in 5% CO₂ in air for 48 hours, then MTT
(Sigma Chemicals), which reduces to a coloured formazan in viable cells, (5mg/ml
in PBS, filter sterilised), was added to each well at 20μl/well and the plate again incubated as above. After 4 hours, 100μl/well of 10% SDS/0.01N HCl was added, and the culture left overnight at 37°C in 5% CO₂ in air. The optical density of the wells was read at 560nm on an ELISA plate reader (Titertek Multiscan Mk II, Labsystems, Finland).

C.2.3 RESULTS

Experiment 1: (figure 1)

A mixed lymphocyte culture was set up between irradiated spleen cells from the C57black/10 mouse strain as the stimulator cells and BALB/c lymph node cells as the responder population. Cells were harvested at days 2, 3 and 4 to assess the kinetics of the lymphocyte response with and without the presence of the antisense oligos IL-2as, IL-2Ras and B7as. The control oligo used was IL-2ns. All the oligos were added at a concentration of 10μM.

Without oligonucleotide, there was a daily rise in cell proliferation (as measured by ³H Thymidine incorporation) to a peak of 6950cpm on day 4.

With IL-2as there was a similar baseline at day 2, but a more gentle rise in cell proliferation to a peak of 3020cpm at day 4.

With IL-2Ras a more intermediate response was seen, with a similar baseline again at day 2, and a rise to a peak of 4980cpm by day 4.
With B7as the most dramatic response was seen, with the lowest baseline of all the cultures, seen on day 2, and a virtually flat response thereafter, with a peak proliferation count of 1110cpm on day 3.

The control oligo, IL-2ns, showed a paradoxical response, with a high baseline at day 2 of 5010cpm, subsequently falling to 1900cpm by day 4.

BALB/c lymphocytes alone, as expected, did not proliferate at all.

Thus, overall, there was an increase in proliferation of the control culture through days 2 to 4, with a decrease in proliferation on the equivalent days by all 3 antisense oligos, B7as having the most dramatic effect. The nonsense oligo, however, showed a mildly stimulatory effect on day 2, a phenomenon also noted in some subsequent experiments (figures 4, 5, 6).
Figure 1: MLC- BALB/c vs. C57black

- BALB/c alone
- MLC-no oligo
- IL-2ns
- IL-2as
- IL-2Ras
- B7as

CPM values are shown for each condition, with error bars indicating variability. The bars are color-coded for different days: day 1 (light blue), day 2 (dark red), day 3 (light red).
Experiment 2: (figure 2)

To determine whether the nonsense oligonucleotide sequence we were using was affecting the behaviour of the culture by non-specific mechanisms, we designed two further nonsense oligos; B7ns is composed of the same base sequence as the B7as but in the reverse (3’ to 5’) direction; IL-2nsj is composed of the same bases as the IL-2as, put together in a random sequence.

A mixed lymphocyte culture was set up as above, between irradiated C57black/10 spleen cells as stimulator and BALB/c lymphocytes as responder. Separately, BALB/c lymphocytes were also set up to be stimulated with concanavalin A. The oligos were used at a concentration of 10µM, and the cultures harvested on day 4.

With BALB/c lymphocytes in culture alone, there was minimal proliferation without added oligo, and a similar count level with all three nonsense oligos. When antisense oligos were used, there was no proliferation whatsoever.

With concanavalin A stimulated lymphocytes, there was strong proliferation in the absence of any oligo, to a level of 48,520cpm.

With the addition of the nonsense oligos to the culture, the level of proliferation was reduced to between 10,000cpm (IL-2nsj) and 18,500cpm (IL-2ns). The use of antisense oligos was dramatic in the case of IL-2as and IL-2Ras, with virtually no proliferation, although the level of proliferation with B7as in this case was similar to the control oligos.

In the MLC, the level of proliferation without oligonucleotide was 10060cpm. The addition of B7ns oligo attenuated this to 6140cpm. The addition of IL-2nsj reduced the count to 4970cpm, and a further reduction was seen with the use of
IL-2ns, with a count of 2740cpm. Antisense oligos in this culture completely eliminated cell proliferation.

This experiment was encouraging in that the control oligos were behaving more predictably, and it was seen that they all had some (presumably non-specific) effect on attenuating proliferation. The antisense oligos again showed a significant reduction in proliferation, compared to both the no oligo and nonsense oligo cultures, although this time the B7 antisense effect was not as dramatic as previously.
Figure 2: Con A stimulation of lymphocytes, and MLC

- no oligo
- B7ns
- IL-2nsj
- IL-2ns
- B7as
- IL-2as
- IL-2Ras

BALB/c + medium
BALB/c + conA
BALB/c + C57black
Experiment 3: (figure 3)

Clearly, from the previous experiment, the addition of nonsense oligos was still having a non-specific (possibly toxic) effect on the cell proliferation, although the antisense oligos have the potential to reduce proliferation by up to 90%. For this reason, we decided to analyse this non-specific effect, again by diluting the oligo concentration, in order to assess whether there is an optimal concentration that balances between specific and non-specific effects.

BALB/c lymphocytes were stimulated with concanavalin A, and the three antisense oligos above, as well as the three nonsense oligos already used, were added at decreasing concentrations, 10μM, 2μM and 0.4μM. Cells were harvested on day 3. Proliferation without the addition of oligo was 103,000cpm.

With the addition of IL-2ns at 0.4μM, proliferation was counted at 93,000cpm, but decreased to 46,000 and 8,500 at concentrations of 2μM and 10μM respectively.

With the addition of B7ns, there was a similar dose dependent effect, with counts decreasing from 118,000cpm to 66,500cpm to 34,500cpm at concentrations of 0.4μM, 2μM and 10μM respectively.

Again, with the addition of IL-2nsj, the picture was similar, with counts of 85,500cpm, 42,500cpm and 21,000cpm at concentrations of 0.4μM, 2μM and 10μM respectively.

Use of the antisense oligos showed a similar dose dependent effect, with no significant difference in the amount that cell proliferation was reduced, compared to the nonsense oligos.

IL-2as reduced proliferation from 107,000cpm to 71,500cpm to 44,000cpm at concentrations of 0.4μM, 2μM and 10μM respectively.
IL-2Ras reduced cell proliferation from 100,000 cpm to 60,500 cpm to 22,500 cpm at concentrations of 0.4 μM, 2 μM and 10 μM respectively.

B7as this time was the least impressive, with counts reduced from 123,000 cpm to 115,500 cpm to 67,000 cpm at concentrations of 0.4 μM, 2 μM and 10 μM respectively.

This experiment was unexpected in the relatively modest antisense effect compared to previous results. It demonstrated a dose dependent response to all the oligos, which is relatively linear, but no significant difference between nonsense and antisense oligos. With all the oligos, a concentration of 0.4 μM had no significant effect on cell proliferation.
Figure 3: BALB/c lymphocytes + con A

- 10 micromoles
- 2 micromoles
- 0.4 micromoles

cpm
Experiment 4: (figure 4)

Finally, the effect of different strain combinations was examined, both to achieve a stronger MLC response on which to test the oligos, and also to check that the oligo effect (antisense or nonsense) was not strain-specific.

A mixed lymphocyte culture was set up between irradiated spleen cells from the C3H (Hma-2^h) mouse strain, and lymphocytes from the Balb/c (H-2^d) strain. Balb/c lymphocytes were also stimulated in culture with concanavalin A. B7as and B7ns oligos were added at concentrations of 2μM and 10μM. The cells were harvested on day 4 of culture.

Balb/c cells alone showed minimal proliferation, with or without oligonucleotide, although with a small reduction in the antisense treated cells compared to the nonsense or untreated cells.

There was, as expected, no cell proliferation of the irradiated cells alone.

With the addition of concanavalin A to the Balb/c lymphocytes, there was strong proliferation of the culture without oligo, to 33,400cpm.

In the mixed lymphocyte culture, the proliferation without oligo was 23,800cpm.

This experiment reinforced the impression that there is both a specific effect on cell proliferation with the antisense oligos, in addition to a non-specific effect of the nonsense (and possibly the antisense) oligos, which, under some conditions, can also be seen to enhance proliferation.

B7ns appeared modestly stimulatory in both cultures at 2μM, although at 10μM this decreased to roughly half the proliferation level of the control (no oligo) cultures. B7as showed more marked reductions at both 2μM and 10μM, with a maximum reduction in the con A culture of 71% compared to the control culture.
Figure 4: con A stimulated lymphocytes, and MLC

- BALB/c + med
- C3H + med
- BALB/c + con A
- BALB/c + C3H

- no oligo
- B7as 10 micromoles
- B7as 2 micromoles
- B7ns 10 micromoles
- B7ns 2 micromoles
The next stage of experiments was done with the use of lipofectamine, a polycationic liposome suitable for the transfection of DNA into cultured eukaryotic cells. Lipofectamine has been shown to increase the cellular uptake and biological action of antisense oligonucleotides by several fold compared to both monocationic lipids and no lipid (e.g. Zhou et al. 1994).

Two new mouse antisense oligonucleotides were designed, both specific to regions on the mouse IL-2 gene sequence, and designated mIL-2as/2 and mIL-2as/3. Control oligonucleotides used were nonsense (IL-2ns), and an antisense oligo specific to the rat IL-2 gene sequence, RIL-2, known to inhibit rat lymphocyte proliferation in culture (S. Yun, personal communication).

The system used was a culture of lymph node cells from the Balb/c mouse strain, stimulated with con A. Control cultures had no oligo or lipofectamine, or lipofectamine alone, in the incubation.

**Experiment 5: (figure 5)**

Cultures were set up with con A, and harvested on days 1, 2, 3 and 4. Lipofectamine was used at a concentration of 10µg/ml, and oligos were added to a concentration of 1µM.

Without addition of oligo or lipofectamine, there was cell proliferation increasing through days 1 to 3, and coming back down slightly by day 4.

Lipofectamine alone caused a slight reduction in proliferation, on all days.

Lipofectamine with nonsense oligo showed a very slight increase in proliferation on days 2, 3 and 4.
Lipofectamine with IL-2as/2 again showed a slight increase in proliferation, compared to the unmodified culture.

Lipofectamine with IL-2as/3 showed a dramatic reduction in proliferation, from approximately 50% reduction on day 1, to greater than 90% reduction on days 2, 3 and 4.

Thus IL-2as/3 caused a reduction in proliferation not mirrored by the controls, and also not seen with the IL-2as/2 oligo, suggesting a sequence specific effect. There was no significant toxicity with lipofectamine at the dose used.
Figure 5: Con A stimulated BALB/c lymphocytes

- no addition
- lipofectamine alone
- lipofectamine+IL-2ns
- lipofectamine+IL-2as/2
- lipofectamine+IL-2as/3

Days: day 1, day 2, day 3, day 4
**Experiment 6: (figure 6)**

To further confirm the specificity of action of IL-2as/3, a lymphocyte culture was set up as above, this time using additional controls, rat IL-2 antisense oligo (RIL-2as) and rat IL-2 nonsense oligo (RIL-2ns).

Oligos were all used at a concentration of 1μM, and lipofectamine at10μg/ml, and cultures were harvested on days 3 and 4.

The results mirrored the above experiment, with the new controls showing a minimal difference from the unmodified culture, and mouse IL-2as/3 oligo significantly decreasing cell proliferation.

Thus, with the use of lipofectamine, we were able to reduce the dose of oligonucleotide in the experiment to 1μM, and thereby minimised the apparent toxicity of these agents seen in earlier experiments.
Figure 6: Con A stimulation of BALB/c lymphocytes

- no addition
- lipofectamine alone
- lipofectamine+rIL-2as
- lipofectamine+rIL-2ns
- lipofectamine+IL-2as/2
- lipofectamine+IL-2as/3

cpm

day 3

day 4
To check the level of reduction of the IL-2 protein product in these cultures, an IL-2-dependent cell line, CTLL cells, was used. To these cells were added supernatants from the respective stimulated cell cultures, and subsequent proliferation of the CTLL cells was observed by measuring optical density to assay viable cells.

Experiment 7: (figure 7)
The supernatants from the above experiment (6) were taken, and added to CTLL cells in culture. CTLL cells alone, to which exogenous IL-2 had been added, provided a baseline for comparison. The optical densities at serial dilutions were read at day 3 and day 4.

All the controls (to which supernatants had been added), as well as the antisense oligo IL-2a/s2 fell within a consistent narrow band of readings at roughly half the level of the CTLL control. IL-2a/s3, in line with the cell proliferation data, appeared to produce far less IL-2, with an O.D. line well below the controls.

These data indicate that the antisense effect with IL-2as/3 seen in the cell proliferation assays, is supported by a demonstration of diminished target protein.
Figure 7: CTLL proliferation assay, to measure amount of IL-2 in supernatants from lipofectamine + oligo treated cultures. Day 2 and day 3 supernatants tested.
IL-2 production measured by CTLL cell proliferation assay - Day 2
IL-2 production measured by CTLL cell proliferation assay - Day 3
Antisense oligonucleotides in theory are an elegant and attractive way of blocking gene expression in a particularly specific manner. However, the practicalities have not so far been as clear-cut as the theory, and it has become increasingly apparent that the presence of non-specific effects necessitates careful analysis, with measurement of more than one end-point.

The inhibition of T-cell co-stimulation molecules using monoclonal antibodies is established as an effective means of prolonging experimental transplant survival, and it would seem appropriate to use antisense technology to achieve the same effects. The main use of antisense oligos to date has been to inhibit the expression of viral genes or oncogenes. The experiments reported in this chapter have been to assess whether these agents can suppress the expression of cytokine or co-stimulatory molecule genes in the context of T-cell stimulation. The mouse model was chosen with the long-term aim of utilising effective oligos in in vivo transplant experiments.

In the initial set of experiments a dramatic effect is seen on cell proliferation with certain antisense oligos directed at different cytokine genes. The effect is seen to be dose-dependent and active in both MLC and con A stimulated cells. The nonsense controls demonstrated a non-specific suppressive effect on some of the cultures (figs. 1 and 2). New nonsense oligos were designed, which reinforced the impression that there is a toxic component to the effect of these oligos at the higher doses (figs. 3 and 4).
These initial experiments encouraged us to investigate the use of cationic liposomes in the cultures, which facilitate endocytic exit of the oligos, and concomitantly reduce the concentration of oligo required by at least a factor of ten. The IL-2 molecule was chosen as the target, and one of two antisense oligos tested greatly reduced cell proliferation, with control oligos at the same concentration having no discernible effect on proliferation (figs. 5 and 6). In line with the requirement for rigorous demonstration of a specific antisense effect, the level of target protein was assessed in these cultures, and was consistent with the effect on cell proliferation. These studies imply that if low enough doses of oligo can be used, combined with high efficiency of uptake, then specificity of effect can more readily be achieved.

The ability of antisense oligonucleotides to produce sequence specific gene inhibition is now well established, but the critical evaluation of their effectiveness is fraught with pitfalls, necessitating rigorous understanding and use of experimental controls. These experiments have utilised a number of methods to establish the presence of an antisense effect, including cell permeabilisation, demonstration of a biological effect and demonstration of reduction of the target protein. The interpretation of any antisense study depends on the strength of its controls, as conclusive proof of absolute specificity is rarely achievable. The results presented here are strongly suggestive of a gene-specific effect, and establish that it is feasible to consider this technology as an alternative means to monoclonal antibodies, to inhibit cytokines in transplant rejection.
C.3 A REPORT AND STUDY OF MHC CLASS II DEFICIENT PATIENTS

C.3.1 INTRODUCTION

The major histocompatibility complex (MHC) class II molecule is a cell surface molecule which plays a pivotal role in the presentation of foreign antigen to the host immune system (Kappler et al. 1976). The lack of expression of this molecule, described in a number of patients, results in a variable combined T and B lymphocyte immunodeficiency (Mach et al. 1994). Perhaps more crucially it provides an inherent model for understanding the mechanism of MHC class II regulation and has already provided some striking insights in unravelling the factors involved.

Using complementation studies on cell lines from MHC class II deficient patients, at least four distinct mutations have been recognised so far, involving regulatory proteins acting on the class II promotor region (Benichou et al. 1991). Those regulatory factors so far elucidated show remarkable specificity for MHC class II expression, yet there remains a great deal of information to be unveiled before the regulatory jig-saw is complete.

There have been no reported clinical features that distinguish patients from different complementation groups. The ethnic grouping of the patients reported to date has been relatively confined, to Spain, North Africa, parts of Northern Europe
and Turkey (Griscelli et al. 1989), indicating a limited source of mutations. In fact the ethnic bias is more marked, in that four of five families in the CIITA deficient group (designated group A) are hispanic, while the majority of patients in group B (genetic defect unknown) are of North African or Turkish origin (Lisowska Grospierre et al. 1994). Herein are presented clinical and immunological data from the first patients to be reported in this country, with particular reference to their ethnic backgrounds, being dissimilar to the currently known groups. In addition, using immunohistology we have studied fibroblast cell lines from three of these patients, for expression of MHC class I, class II and the invariant chain, and report the findings.

C.3.2 MATERIALS AND METHODS

C.3.2.1 Lymphoproliferation to PHA, candida and PPD

Lymphocytes were separated from whole blood in EDTA by density gradient centrifugation (Histopaque-1077, Sigma Diagnostics, Poole, UK). The cells were washed three times and re-suspended at 1x10⁶ cells/ml in RPMI 1640 medium (Gibco Ltd., Paisley, UK) containing 20% autologous serum, 100U/ml Penicillin, 100U/ml streptomycin and 2mmol/L L-glutamine. Viability of the cells was checked with trypan blue. Triplicate cultures containing 100μl (1x10⁵) cells were plated onto a 96-well round bottomed plate (Sterilin, Feltham, England) together with PHA, Candida or PPD antigen, and incubated at 37°C in a humidified 5% CO₂ atmosphere before being pulsed with 1.5μCi ³H Thymidine per well for 20 hours.
Cells were harvested onto glass-fibre paper using an automatic harvester and counted using a betaplate counter (LKB, Bromma, Sweden).

C.3.2.3 T-lymphocyte subsets

Five µl of monoclonal antibody was mixed with 100µl of EDTA blood for 10 minutes. The monoclonal antibodies were labelled with fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE). Negative control antibodies labelled with FITC and RPE fluorochromes were used to check the levels of background autofluorescence and non-specific staining.

C.3.2.4 Monoclonal Antibodies used


This is a mouse IgG1 monoclonal antibody directed against the human Thy-1 molecule.

2. Clonab IN2 (Biotest, Dreieich, Germany). A monoclonal mouse IgG1 antibody recognising a 31 kD antigenic component of human invariant chain (Koch et al. 1982), was used on human tissue sections, at a dilution of 1:10

3. The W6/32 antibody to human MHC class I antigen (Barnstable et al. 1978) was a kind gift of Professor A.F. Williams (MRC Cellular Immunology Unit, Oxford, UK), and was used as partially purified immune ascites at 1/100 of the saturating dilution.
4. The F16-27-12 antibody to human class II antigen (Fuggle et al. 1983) was a kind gift of Dr. S.V. Fuggle (Nuffield Department of Surgery, Oxford, UK), and was also used as partially purified immune ascites at 1/100 of the saturating dilution.

5. Peroxidase conjugated rabbit anti-mouse (RAM) antibody was purchased from Dako Ltd., Bucks., UK. Recombinant human interferon-γ was a kind gift of Dr. N. Klein (Institute of Child Health, London).

C.3.2.5 Freezing of cells

I) Freezing mixture:
10% DMSO (dimethyl sulphoxide, Sigma) in RPMI 1640 Hepes buffered medium, supplemented with 100 units/ml penicillin, 100 μg/ml Streptomycin, 2 mM glutamine, and 10% foetal calf serum.

II) Procedure:
Cells (5x10^6 minimum) were centrifuged at 400g for 10 minutes at 4°C. The cells were gently resuspended to 10^6/ml in cold freezing mixture and control rate frozen in liquid nitrogen at approximately 1°C per minute to -70°C, then transferred for storage in the vapour phase of liquid nitrogen.
C.3.2.6 Thawing and culture of fibroblast cell lines

Primary fibroblast cell lines from 3 of the patients (unrelated) were used, designated patients ED, RS and VH, along with fibroblasts from a control (non MHC class II deficient) patient, designated JW.

The appropriate human fibroblast cell line was retrieved from liquid nitrogen storage, thawed and immediately diluted in cold Hepes medium. After 2 washes at 4°C, the cells were suspended in RPMI 1640 medium, supplemented with 10% FCS, and allowed to multiply to confluence in 75cm² plastic flasks (Becton Dickinson, NJ, USA) at 37°C in 5% CO₂. Once confluent, the fibroblasts were rendered non-adherent by removing the medium followed by the addition of 3mls of 0.25% trypsin, and incubating at 37°C in 5% CO₂ for 5 minutes. The cells were then centrifuged at 1000rpm for 5 minutes, and re-suspended at half the original concentration in the above medium.

C.3.2.7 Growth of fibroblasts on chamber slides

Eight-well glass chamber slides (Nunc Inc., Illinois, USA) were pre-coated with fibronectin (Sigma Chemicals) to facilitate adherence of the fibroblasts. Fibronectin at 5µg/ml was put in each well (approx. 200µl/well) and left for 15 minutes. This was then removed and the slides left to air-dry for 15 minutes. The suspended cells were then plated into each well (0.45mls/well) either with or without the presence of human IFNγ added to the medium at a concentration of 20ng/ml.

The fibroblasts were left to grow at 37°C in 5% CO₂ for 72 hours at which point they had reached confluence.
C.3.2.8 Immunoperoxidase staining of fibroblasts

(The following procedures were carried out at room temperature unless otherwise stated)

Fixing cells

The growth medium was removed, and the wells briefly washed with phosphate buffered saline (PBS). 200μl of acetone (Sigma Chemicals) was added to each well and left for 15 minutes, followed by two more washes with PBS.

Permeabilisation of cells

Half of the wells studied were permeabilised by the addition of 0.1% Triton (Sigma Chemicals) made up in PBS to each well and left to stand for 2 minutes. The wells were then washed twice with PBS.

Application of Antibody

50μl of antibody at the appropriate (saturating) dilution was applied to each well and incubated for 30 minutes in a humidified chamber. The antibody was then removed by tipping off, and the wells washed twice in PBS for a total of 3 minutes of soaking. 50μl of peroxidase conjugated RAM antibody (at a 1/20 dilution) was added to each well and incubated for 30 minutes in a humidified chamber. The wells were then washed twice in warm (37°C) PBS.

Developing of enzyme

The peroxidase enzyme was developed with a solution of diaminobenzidine (DAB) at 0.6mg/ml with 0.03% hydrogen peroxide in PBS. 50μl of the solution was added to each well and incubated at 37°C for 10 minutes, until the sections had
gone visibly brown. The plastic well surrounds were then removed, and the slides washed in PBS followed by water.

**Counterstaining the sections**

The slides were placed in filtered Harris Haematoxylin for 30 seconds and washed in water. They were mounted with glass cover slips and were then ready for scrutiny under the microscope.

**C.3.3 RESULTS**

**C.3.3.1 Patient data** (Tables 1 and 2)

Five children presented over a 7 year period, 1 male and 4 females. The mean age of first serious infection was 5 months (median 4 months). Two patients were of British caucasian origin and 3 were from the Indian subcontinent. All suffered from repeated severe infections, predominantly viral and fungal, and 3 from *pneumocystis carinii* pneumonia. Three had protracted diarrhoea and failure to thrive, one of these children having a jejunal biopsy suggestive of an auto-immune enteropathy. Two children contracted central nervous system (CNS) enterovirus infection. Three children died, at 6 months, 6.5 years and 7.3 years. One patient survives aged 2.9 years, following a successful unrelated bone marrow transplant (BMT), having rejected an earlier parental BMT. The other is now aged 14.3 and is stable on maintenance treatment. He has not required a BMT.
Laboratory investigations show 4 patients negative for MHC class II expression on peripheral blood mononuclear cells, 1 had less than 1% of normal expression. MHC class I expression was variably affected. CD4 count was 12-43% (normal 45-65%), CD8 was 11-58% (normal 15-25%). In vitro antigen stimulation tests (to candida, PHA and PPD) were almost universally poor whenever tested. Humoral immunity, as measured by serum immunoglobulin levels, was severely impaired; 4 patients had a decrease in at least two immunoglobulin isotypes.
<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age at Presentation</th>
<th>Presenting symptoms</th>
<th>Country of origin</th>
<th>BMT</th>
<th>Outcome</th>
<th>Predominant infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH</td>
<td>F</td>
<td>4 months</td>
<td>Chickenpox</td>
<td>British</td>
<td>Yes</td>
<td>Alive at 2.9 years</td>
<td>E.coli meningitis, Pneumocystis carinii pneumonia, CNS enterovirus</td>
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<tr>
<td>ED</td>
<td>F</td>
<td>5 months</td>
<td>E.coli meningitis</td>
<td>British</td>
<td>No</td>
<td>Died at 7.5 years</td>
<td>Chickenpox, Interstitial pneumonitis, Widespread candida</td>
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<tr>
<td>RS</td>
<td>F</td>
<td>4 months</td>
<td>Failure to thrive, Persistent cough, Oral candida</td>
<td>Pakistan</td>
<td>No</td>
<td>Died at 6 months</td>
<td>RSV pneumonia, Persistent diarrhoea, Widespread candida</td>
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<tr>
<td>FZ</td>
<td>M</td>
<td>2 weeks</td>
<td>Persistent oral candida</td>
<td>Pakistan</td>
<td>No</td>
<td>Alive at 12.5 years</td>
<td>Chickenpox, CNS echovirus, adenovirus, measles, Respiratory cytomegalovirus, Measles</td>
</tr>
<tr>
<td>NZ</td>
<td>F</td>
<td>12 months</td>
<td>Repeated ear infections, Chronic maxillary sinusitis</td>
<td>Pakistan</td>
<td>No</td>
<td>Died at 6.5 years</td>
<td>Stool cryptosporidium Chickenpox, Systemic aspergillosis</td>
</tr>
</tbody>
</table>
TABLE 2: Summary of immunological investigations on patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>CD4 count at presentation (%)</th>
<th>CD8 count at presentation (%)</th>
<th>MHC class I expression (%)</th>
<th>MHC class II expression (%)</th>
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</thead>
<tbody>
<tr>
<td>VH</td>
<td>27</td>
<td>21</td>
<td>89</td>
<td>NIL</td>
</tr>
<tr>
<td>ED</td>
<td>16</td>
<td>23</td>
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<td>RS</td>
<td>14</td>
<td>35</td>
<td>54</td>
<td>NIL</td>
</tr>
<tr>
<td>FZ</td>
<td>12</td>
<td>62</td>
<td>ND</td>
<td>&lt; 1</td>
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<tr>
<td>NZ</td>
<td>21</td>
<td>19</td>
<td>ND</td>
<td>NIL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients</th>
<th>PHA stimulation index</th>
<th>Candida stimulation index</th>
<th>PPD stimulation index</th>
<th>IgG (g/l)</th>
<th>IgM (g/l)</th>
<th>IgA (g/l)</th>
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<tbody>
<tr>
<td>VH</td>
<td>414</td>
<td>8.6 (control 26.1)</td>
<td>7.7 (control 27.1)</td>
<td>6</td>
<td>8</td>
<td>1.3</td>
</tr>
<tr>
<td>ED</td>
<td>9</td>
<td>0.8 (control 54.8)</td>
<td>0.9 (control 78.1)</td>
<td>83</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>RS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NIL</td>
<td>51</td>
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<td>ND</td>
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<td>10</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>
C.3.3.2 Immunohistology on fibroblast cell lines

Thy-1 antibody staining (Figure 1)

This antibody was used as a positive control for all the cell lines, as it is known to stain human fibroblasts strongly. As expected, staining was very strongly positive on all the cell lines, with the whole section taking up a deep brown stain. There was no difference between IFNγ treated and untreated cells, and no difference with permeabilisation on any of the sections.

MHC class I expression (Figure 1)

MHC class I, as expected, shows a basal level of expression on unstimulated cells, which is upregulated in both the patients' and control cell lines, with the addition of interferon-γ. There is no appreciable difference between permeabilised and non-permeabilised cells for any of the sections. Patient ED is moderately positive without IFNγ, and strongly positive with IFNγ.

Patient RS is also positively stained without IFNγ, and more strongly positive with IFNγ, though with slightly weaker upregulation than that seen with ED.

Patient VH displays similar levels of expression to RS, both with and without IFNγ.

Control JW is more strongly stained for class I on the non-IFNγ section than any of the patients, and similarly markedly upregulated with IFNγ.
MHC class II expression (Figure 2)

For MHC class II, we expected to see no expression on the patients cells, most obviously because these children have been shown to be completely deficient for class II expression on lymphoid cells. Secondly, class II is not normally expressed on resting fibroblasts, although IFNγ stimulated fibroblasts show class II expression. In fact, all the patients fibroblasts showed a very weak staining, with no significant difference between IFNγ treated and non-treated groups. The control cells were more strongly stained. Again, permeabilisation had no effect on visible expression on any of the sections.

The most likely explanation for this unexpected positive staining is that there is an element of non-specific binding of this antibody to epitope(s) on human fibroblasts.

Patient ED displayed weakly positive staining for both IFNγ positively and negatively treated sections, the level of staining being considerably less than that for MHC class I expression in the same patient.

Patient RS displayed an even weaker positive staining in both IFNγ, treated and untreated sections.

Patient VH showed the weakest staining, only just discernible on both IFNγ treated and untreated cells.
Control JW was slightly more positive for class II expression than any of the patient cells, with perhaps a marginal increase in staining of the IFNγ treated cells. The staining was not as strong as that for MHC class I in the same cells.

Invariant chain expression (Figure 3)

Conceivably the most interesting observation was that of invariant chain expression on these cells. All the patients' fibroblasts were completely negative for Ii, both with and without IFNγ, whereas in the control JW cells we demonstrated Ii expression at a level similar to MHC class I expression (non-IFNγ treated) in the same cells. There was a slight increase in Ii expression on control cells with IFNγ treatment. No difference was seen on any of the sections with permeabilisation.
This page (opposite):

Figure 1:

Expression of **MHC class I** in fibroblasts from

(a) control patient, without IFN-γ stimulation,

(d) control patient, with IFN-γ stimulation,

(b) MHC class II deficient patient, RS, without IFN-γ stimulation,

(e) MHC class II deficient patient, RS, with IFN-γ stimulation.

Expression of **Thy-1 control** in fibroblasts from

(c) control patient, without IFN-γ stimulation,

(f) MHC class II deficient patient, RS, without IFN-γ stimulation.

Following page:

Figure 2:

Expression of **MHC class II** in fibroblasts from

(a) control patient, without IFN-γ stimulation,

(b) control patient, with IFN-γ stimulation,

(c) MHC class II deficient patient, RS, without IFN-γ stimulation,

(d) MHC class II deficient patient, RS, with IFN-γ stimulation.

Following page:

Figure 3:

Expression of **invariant chain** in fibroblasts from

(a) control patient, without IFN-γ stimulation,

(c) control patient, with IFN-γ stimulation,

(b) MHC class II deficient patient, RS, without IFN-γ stimulation,

(d) MHC class II deficient patient, RS, with IFN-γ stimulation.
C.3.4 DISCUSSION

MHC class II deficiency is a rare, autosomal recessive disease whose detection requires a high level of clinical awareness, and which has implications in elucidating important biological processes. MHC class II molecules are expressed on the surface of antigen presenting cells, and present foreign antigen to T lymphocytes in the initiation of an immune response. They also play a role in trans-membrane signalling and in selection of the T cell repertoire in thymic ontogeny. Mice lacking MHC class II molecules show near complete elimination of CD4⁺ T lymphocytes, although a substantial number of CD4 single positive cells reside in the thymus (Cosgrove et al. 1991).

The regulation of class II gene expression is very tightly controlled, as would be expected for such a central molecule of the immune system (Janeway et al. 1984). Control of regulation of expression is important in auto-immune disease (e.g. Bottazzo et al. 1983) and graft rejection. Several transcription factors are now known to bind to the promotore region of the class II gene (e.g. RFX, NF-Y, X2BP), and their interaction is complex and incompletely understood (Reith et al. 1994). Study of class II deficient cell lines has revealed a crucial mediator of transcription, CIITA, from a subset of patients. This nuclear protein appears not to bind directly to the class II promoter region, but to be a specific controller of all the regulatory factors (Steimle et al. 1993). In another group, a defect in a subunit of the RFX complex, another regulating protein, has been identified (Reith et al. 1988). Recently, twin boys have been reported with low levels of one class II
isotype (and absence of the other two isotypes) (Wolf et al. 1995), indicating perhaps a previously unrecognised defect in the way the regulatory factors interact, or a leaky phenotype as in one of our patients. These boys, though yet at a young age, appear to show a mild phenotype.

The clinical features of our patients described largely mirrors the phenotype of the patients reported in the literature, with severity of immunodeficiency not apparently related to the type of molecular defect. There is, though, a high rate of early mortality in reported series (23/39) with survivors requiring BMT(14/39) and the few patients without BMT remaining in a poor condition despite considerable medical care (Griscelli et al. 1989). One of our patients, however, appears to display a mild presentation and is stable without having had a bone marrow transplant. This patient, of Pakistani origin, has low CD4 and Ig counts, and has had repeated infections in early childhood but is now stable at 14.5 years of age. His relative well-being may be ascribed to having a low residual level of class II expression (<1%). Another patient, of British caucasian origin was well until 7.3 years of age. She, similarly had severe infections at an early age but did not require a BMT, and clinically appeared to stabilise. She had no detectable class II expression but near normal CD4 count (43%). Interestingly, both these patients suffered from CNS echovirus infections (to which the latter succumbed), a complication not noted in the literature.

The patients reported so far appear to originate from a limited genetic pool, coming from parts of Northern Europe, Spain, North Africa and Turkey. The specific complementation groups also appear to segregate to specific geographic
areas (LisowskaGrospierre et al. 1994), implying a limited number of original mutations. The patients we report come from Britain and the Indian subcontinent, two areas from which this disease has not previously been recognised. This raises the possibility of new mutations, which clearly would add to our knowledge of MHC class II gene regulation.

The immunohistology data was obtained as we began to further characterise these children with regard to their specific molecular defects. The weak positivity in staining fibroblasts with MHC class II antibody is likely to result from cross-reactivity of the antibody, as it would be unexpected even for normal unstimulated fibroblasts to be expressing class II. This is supported by there being minimal difference between control cells and patient cells in class II expression. It is worth bearing in mind, however, that there are reports of HLA-DR positivity in non-lymphoid cells of a class II deficient patient (Schuurman et al. 1995) and in B cells of the twin boys mentioned above (Wolf et al. 1995), implying that the defect in class II antigen expression is not necessarily present to the same extent in cells of different lineages, and may indeed be under the control of different regulatory genes.

The complete lack of invariant chain expression in the patient’s fibroblasts is perhaps more intriguing. It is induced by IFNγ, and would be expected to be expressed on the stimulated fibroblasts, as seen on the control cells. CIITA has been shown to be necessary for Ii transcription, and although it is conceivable that all three patients are CIITA deficient, this is improbable given that numerically CIITA deficiency is a minor subset of the reported complementation groups.
Normal levels of Ii were seen in B cells of the twin boys analysed above, although their complementation group is not known. In the other complementation groups the expression of invariant chain has not been studied, making it difficult to know whether other MHC class II transcription factors also regulate Ii expression. Our results indicate that this is likely, given that CIITA is the only factor so far to be shown to regulate Ii expression, and that our patients are unlikely all to be CIITA deficient.

Recognition of MHC class II deficiency requires a high level of clinical awareness, particularly if, for example, the total B cell count is low, making the interpretation of class II expression on B cells more difficult. There is little doubt that cases have not been recognised in this country in the past. Understanding the disease at a molecular level yields the potential for future gene therapy. On a wider basis, the condition is instructive in how the study of a single gene defect can lead to unique insights into basic immune mechanisms. The MHC class II molecule is central to the manner in which the immune system deals with foreign antigens, and understanding its control will be a crucial step in understanding the immunopathogenesis of a large number of diseases.
C.4 \textbf{A SYSTEMATIC STUDY OF INVARIANT CHAIN AND MHC CLASS II EXPRESSION IN RAT TISSUES}

C.4.1 \textbf{INTRODUCTION}

The invariant chain (li) is known to play an important role in the assembly, intracellular transport and peptide loading of MHC class II molecules. In the absence of invariant chain, cell surface expression of class II MHC molecules is generally diminished, and, of particular importance, the spectrum of class II associated peptides can be markedly altered, with a skew towards cytosolic rather than extracellular peptides (e.g. Momburg et al. 1993; Bodmer et al. 1994). Thus, the expression of MHC class II molecules alone or coordinately with invariant chain will influence the capacity of a cell to present peptides, as well as the nature of the peptides presented.

Dendritic cells (DC), originally described in lymphoid organs (Steinman et al. 1973), are known to be the most potent of antigen presenting cells (Steinman et al. 1978). Cells with dendritic morphology and many of the characteristics (e.g. intense MHC class II expression) of lymphoid dendritic cells are found in connective tissues throughout the body (except the central nervous system) and are known as interstitial dendritic cells (Hart et al. 1981b). A similar dendritic cell, the Langerhans cell (LC), is found in the epidermis of skin (Silberberg Sinakin et
Following isolation, both lymphoid DC and LC strongly express both invariant chain and MHC class II molecules and can process and present exogenous antigen (Pure et al. 1990; Kampgen et al. 1991). Interstitial dendritic cells have not been studied in this way, presumably because of the difficulty in isolating these cells.

Using monoclonal antibodies on histological sections we have looked at invariant chain and MHC class II expression on rat tissues to compare the relative expression of these two molecules, particularly in structures which may be relevant to antigen presentation such as the renal tubular epithelium and small intestinal epithelium, and also in interstitial dendritic cells, with particular reference to antigenically secluded tissues such as the heart.

We were surprised to find that in certain tissues, invariant chain expression did not, as expected, correlate with class II expression. The level of staining of class II and of invariant chain was similar in the spleen. However, basal expression of invariant chain in the renal epithelial tubule was not detected, in comparison to ample expression of class II, although II was up-regulated in the transplanted kidney. The small intestinal epithelium in the rat displayed strong class II expression and weak II expression, contrasting with previous findings (Vidal et al. 1993). In the connective tissue of the kidney, heart, liver, trachea and pancreas, class II expression was seen on interstitial dendritic cells. In general, invariant chain in the same tissues was expressed on virtually no cells.
Thus the expression of II in various tissues appears discordant with MHC class II expression, according to the cell type examined, perhaps reflecting the different nature of antigens presented by these cells. There is a growing recognition of the importance of the invariant chain in the physiology of MHC class II molecules and possibly a crucial role in the critical area of self and non-self antigen recognition, and the relevance of these data is discussed.

C.4.2 MATERIALS AND METHODS

C.4.2.1 Animals.

Adult male rats of the DA (RT1\* ) strain and PVG strain were obtained from Harlan-Olac U.K. Ltd. (Bicester, U.K.). Kidneys were transplanted from DA to PVG rats and removed at day 5 for immunohistology. We examined a total of 4 animals for liver, spleen, and kidney graft sections, 3 animals for thymus, pancreas and small intestine sections, 5 animals for heart sections and 12 animals for normal kidney sections. In addition we looked at single sections of trachea and lung.

C.4.2.2 Human tissue samples.

Human spleen was obtained from normal tissue routinely removed from cadaveric kidney donors at the Royal London Hospital, Whitechapel, London. Thymus was obtained from routine thymectomy for access, during paediatric open heart
surgery, at The Hospital For Sick Children, Great Ormond Street, London, WC1N. Kidney samples were acquired at nephrectomy for discrete adenocarcinomas or transitiotional cell carcinomas, by taking a sample from a normal pole, distant from the tumour.

### C.4.2.3 Antibodies

Clonab IN2 (Biotest, Dreieich, Germany), a monoclonal mouse IgG₁ antibody recognising a 31 kD antigenic component of human invariant chain (Koch et al. 1982), was used on human tissue sections, at a dilution of 1:10.

The RG11 mouse IgG₂a monoclonal antibody, directed against a C-terminal γ chain segment of rat invariant chain, was a kind gift of K. Reske (Mainz, Germany) (Fisch et al. 1992). This antibody reacts with all isoforms of rat invariant chain. The antibody was used on rat tissue sections at a dilution of 1:5.

The MRC OX6 cell line secreting IgG₁ antibody to rat RT1-B class II MHC antigens (Barnstable et al. 1978) was a kind gift of Prof AF Williams (Sir William Dunn School of Pathology, Oxford).

F15-42-1 (McKenzie et al. 1981). This is an IgG₁ monoclonal antibody directed against the
human homologue of the Thy-1 molecule, and was used as the negative control antibody.

These antibodies were used as immune ascites partially purified by ion exchange chromatography.

C.4.2.4 Immunohistology

Tissue samples for immunohistology were frozen in liquid nitrogen immediately after harvesting. Cryostat sections of 7μm were cut on to Vectabond ® (Vector Laboratories, Peterborough, Sussex) treated slides, air-dried, fixed in acetone, and stored at -20°C until used. To stain the sections, the slides were thawed, and the appropriate mouse monoclonal antibody was put on the sections at the appropriate dilution. These were incubated at room temperature for 30 minutes and washed twice in Tris-buffered saline. For immunoperoxidase staining, horseradish peroxidase-coupled rabbit anti-mouse Ig (Dako Co., Copenhagen, Denmark) was used at a 1:7 dilution in 20% normal rat serum to block antibodies crossreacting with rat Igs. The second incubation was at room temperature for 30 minutes and the slides were then washed twice. The coloration was developed at 37°C with diaminobenzidine (Sigma Chemical Co., Poole, UK) at 0.6 mg/ml containing 3 μl of 100 vol hydrogen peroxide. Sections were counterstained with Harris’ s haematoxylin.
C.4.2.5 Kidney transplantation in rats.

The technique involved a left orthotopic transplant with end-to-end anastomosis of the renal artery, renal vein and ureter, using microsurgical techniques, essentially as previously described (Fabre et al. 1971). All ischaemia times were less than 30 minutes.

Table 1: Number of animals used for each tissue examined

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>NUMBER OF ANIMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Intestine</td>
<td>3</td>
</tr>
<tr>
<td>Heart</td>
<td>5</td>
</tr>
<tr>
<td>Normal Kidney</td>
<td>12</td>
</tr>
<tr>
<td>Kidney Graft</td>
<td>4</td>
</tr>
<tr>
<td>Spleen</td>
<td>4</td>
</tr>
<tr>
<td>Thymus</td>
<td>1</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3</td>
</tr>
<tr>
<td>Trachea</td>
<td>1</td>
</tr>
</tbody>
</table>
C.4.3 RESULTS

Antibody staining on individual tissues:

Small Intestine

**MHC class II:** The cores of the villi were intensely positive, and on the epithelial cells there were positively expressing cells in the crypts of the villi, with less expression as the tips of the villi were reached. In one of the three rats examined, the whole of the epithelium was intensely class II positive, with invariant chain also showing greater intensity of staining in this animal than in the others.

**Invariant chain:** The cores of the villi were intensely positive for II, at a similar level to class II staining, whereas the epithelium was only weakly positive on the tips of the villus, and negative in the crypts. In the smooth muscle and submucosa there were few positive cells, with no obvious dendritic cells seen.

Heart

**MHC class II:** In the interstitium, there was a considerable scattering of positive cells, assumed to be interstitial dendritic cells (with the same pattern of scattering expected as for IDC's). All five animals examined showed a similar level of expression.

**Invariant chain:** In two of the animals, no li expression was seen, in the others only very occasional positive cells were seen, in one of these the few positive cells tending to be focal on the section, perhaps indicating local inflammation.
Normal kidney

MHC class II: There were class II positive expressing structures throughout the renal cortical and medullary interstitium, similar in all animals. There was also some class II expression seen in the smooth muscle layer of the arterioles. The endothelial layer of the arterioles was class II negative. The proximal renal tubular epithelium was strongly positive in all animals, in contrast to Ii expression.

Invariant chain: In three of the animals there were a few scattered positively stained cells in the interstitium, and in the other two animals there was virtually no Ii expression. The arterioles (smooth muscle and endothelium) appeared to be Ii negative. The renal tubular epithelium in all the animals was completely negative for invariant chain expression.

Kidney grafts

To test if invariant chain expression could be induced on the epithelial cells, DA kidneys were transplanted to PVG rats. By the 5th day after grafting, there is a strong rejection response with a diffuse leucocyte infiltrate and the induction of both class I and class II MHC antigens (Milton et al.1986).

MHC class II: In the day 5 rejecting kidney graft, both medulla and cortex were very strongly positive for class II in the tubules and connective tissue. The vascular endothelium in the arterioles had also become positive at this stage.
Invariant chain: Invariant chain expression had been induced, at almost the same intensity of staining as class II expression, on the tubular epithelium and also on cells in the connective tissue. The arteriolar vascular endothelium had also become li positive, although the vascular smooth muscle layer appeared to remain li negative.

Spleen

MHC class II: The dendritic cells in the T-cell areas were uniformly class II positive, and the B cell areas were also uniformly and intensely positive. The red pulp (macrophages) and marginal zones (macrophages and lymphoid cells) were more weakly positive for class II expression.

Invariant chain: On all the sections seen, li distribution and level of expression was not significantly different from the class II expression described above.

Thymus

MHC class II: The thymic medulla was uniformly positive, with some negative T-cell areas seen in between the positively stained areas. The cortex was also class II positive, including the cortical epithelial cells.

Invariant chain: The medullary area was mostly negative, with some intensely positive areas seen, mainly adjacent to the cortex. The cortex was li positive, though with less intensity than for class II expression.
Lung

**MHC class II**: There were many strongly positive cells scattered throughout the connective tissue, presumably macrophages, with virtually no staining seen on the alveolar epithelium.

**Invariant chain**: This was also expressed throughout the connective tissue, albeit at a much lower frequency compared to MHC class II.

Pancreas

**MHC class II**: Class II expression was seen on a scattering of interstitial cells, as in the heart sections, assumed to be dendritic cells.

**Invariant chain**: This section again mirrored the heart sections, in that there were very few positive cells throughout the sections.

Trachea

**MHC class II**: There were scattered positive cells in the tracheal connective tissue. The epithelium was class II negative.

**Invariant chain**: Again, there was a scattering of positive cells, though with far less frequency than for class II in the interstitium. The epithelial cells were II negative.
Summary of immunohistology data by cell type

*Epithelial cells.* In normal rat small intestine the epithelium was strongly positive for class II expression, more strongly so on the villi and out of the crypt regions than in the crypts themselves. The expression of invariant chain in these cells, however, was only weakly positive. In the renal cortex there was a scattering of class II positive tubular epithelium, whereas these were completely negative for invariant chain. Tracheal epithelium was class II and Ii negative. Thymic cortical epithelium was strongly class II positive, with much less Ii positivity.

*Interstitial Cells.* In the heart and pancreas, there was a similar scattering of class II positive cells in the connective tissue, assumed to be interstitial dendritic cells (Hart et al. 1981b). In the pancreas there was some decrease in invariant chain expression compared to class II, and in the heart invariant chain was virtually absent in the interstitium. In the small intestine, the B-cells, macrophages and interstitial dendritic cells around the crypts were weakly positive for both molecules, class II slightly more so. In the villi, there was strong positivity for both class II and Ii, at a similar level. The lung appeared to show a similar level of staining for both Ii and class II. Tracheal connective tissue exhibited scattered class II positive cells with slightly less invariant chain positive cells. There were a few Ii positive structures scattered through the connective tissue of renal cortex and medulla, similar but with much less frequency than the class II positive structures therein. The spleen showed highly positive staining for class II in the areas of red pulp, and strong positivity in the B cell areas of the white pulp. Invariant chain
staining showed no difference from this in pattern or intensity. In the thymus, as with thymic epithelium, the medullary cells were strongly expressing class II with comparatively much reduced II expression.
This page (opposite):

Figure 1:
Expression of Thy-1 control (a), MHC class II (b) and Invariant chain (c) in rat spleen.
Expression of Thy-1 control (d), MHC class II (e) and Invariant chain (f) in rat heart.

Following page:
Figure 2:
Expression of Thy-1 control (a), MHC class II (b) and Invariant chain (c) in rat normal kidney.
Expression of Thy-1 control (d), MHC class II (e) and Invariant chain (f) in rat kidney graft.

Following page:
Figure 3:
Expression of Thy-1 control (a), MHC class II (b) and Invariant chain (c) in rat small intestine.
Expression of Thy-1 control (d), MHC class II (e) and Invariant chain (f) in rat pancreas.

Following page:
Figure 4:
Expression of Thy-1 control (a), MHC class II (b) and Invariant chain (c) in rat thymus.
C.4.4 DISCUSSION

When it was first discovered, associated with the MHC class II molecule, the invariant chain was thought to be a crucial factor in cell surface expression of the class II molecule. Thereafter, its role within the cell was gradually elucidated, so that it became clear that li is a crucial chaperone to the class II molecule, from synthesis to eventual membrane expression loaded with exogenous peptide for presentation to CD4 T-cells. It has transpired that li plays a part in every step of this pathway through different regions of its polypeptide chain. In the endoplasmic reticulum, once class II chains are synthesised, they associate with li to form a nine subunit complex (Roche et al. 1991b) which prevents endogenous peptides from binding in the peptide binding groove of the class II dimer. The invariant chain possesses three structural transport signals, that operate according to the status of li within the ER. The full length unbound li possesses an ER retention signal, preventing it from leaving before associating with class II. Truncated li has a separate motif dominating which directs it to a degradation compartment. Finally, li bound to class II dimers is directed by a third signal to the endosomal compartment via the golgi complex (Lotteau et al. 1990).

Thus li delivers MHC class II dimers, free of endogenous peptide, to endosomes, which are the site of exogenous antigen breakdown. The obstacle now is that the peptide binding groove is occupied by invariant chain peptides (CLIPs) (e.g. Malcherek et al. 1995). Within the endosomes, acidic conditions and proteolytic activity cleave li from the class II dimers, leaving the class II molecule free to
traffic through the endocytic pathway to capture distinct exogenous peptides (e.g. Castellino et al. 1995). From here, the logical step is for the newly formed class II/exogenous peptide to be transported to the cell surface for presentation to CD4 T-cells. This does indeed occur, but it is also clear that free li and class II/li complex also appear at the cell surface, the purpose of which in antigen presentation is ill-defined (e.g. Koch et al. 1991a).

It has therefore become apparent that the invariant chain is crucial in almost all stages of defining MHC class II function, and serves to distinguish between the roles of MHC class I and class II. At this point, the logical assumption is that the two molecules are inextricably linked, so that cellular expression of class II would be expected to coincide with li expression.

Concurrent with discoveries about its cellular function, the molecular background to li expression and regulation has been determined to a degree. It transpires, in fact, that the promotor regions of the MHC class II gene and li are largely homologous (Brown et al. 1991b), and that IFNγ and CIITA are involved in the induction of expression of both molecules (Chang et al. 1995). These findings added to the impression that expression of the two ought to be co-ordinate. However, there have been a number of observational studies that have noted asynchronous inducibility of li and class II depending on the cell line involved (e.g. Koch et al. 1984). Thus further cell-specific elements are likely to be involved in transcription of the two molecules (Abdulkadir et al. 1995).
This lays the ground for explaining the observations that we have found in systematically studying MHC class II expression and invariant chain expression in a large number of normal rat tissues, and in rat kidney grafts.

We have studied normal sections from rat tissue samples, and found discordant expression, varying according to the structure and organ examined. Incidentally we also studied normal human thymus, spleen and kidney in the same manner, but the interpretation of the findings was confounded by the difficulty in obtaining 'normal' tissue. The kidney samples were obtained from nephrectomies for renal carcinoma, where there was a macroscopically normal looking pole. Three different patient sections were examined, and it was clear that there were areas of abnormal inflammation, as judged by cellular infiltration and patchy increases in MHC class II and Ii expression. Similarly, the human spleen and thymus samples obtained were poor sections, probably suffering from the practical difficulty of not being immediately fresh when cut.

In the rat, interstitial dendritic cells in antigenically secluded organs, such as heart and pancreas, display the expected scattering of class II positive interstitial dendritic cells but virtually no Ii expression. In more 'exposed' organs, such as the small intestine, the level of Ii expression was far more concomitant with the level of class II seen. Epithelial cells of the small intestine and thymus expressed a higher level of class II than Ii. Interestingly, renal tubular epithelium was completely Ii negative, which was a highly unexpected finding in view of the fact
that these cells, unusually for non-‘professional’ APC’s, express class II in the quiescent state.

The epithelial cells of the proximal convoluted tubule of the kidney, uniquely among the parenchymal cells of transplanted organs, normally express class II MHC antigens. The physiological reasons for this expression has remained unclear. Since the expression of invariant chain is likely to influence the capacity of the renal tubular epithelial cells to stimulate allogeneic responses, the absence of invariant chain expression in the quiescent state is an important and unexpected observation. The expression of both class II and invariant chain has been shown to be under the control of CIITA (see section B.2.1), and it may be that there is an additional transcriptional factor in these particular cells that separates the co-ordinate expression of the two molecules. There is conjecture that renal tubular epithelial cells participate in the pathogenesis of auto-immune disease, and the class II associated peptides presented by these cells, self or non-self, would be influenced by the presence of invariant chain.

This perception is re-inforced by similar findings on small intestinal epithelial cells, with only very weak Ii expression demonstrated. Clearly, the small intestinal villi are subject to constant exogenous antigenic stimulus, and the level of invariant chain expressed could play a crucial role in the ability of these cells to present these as foreign antigens to host T cells. This has implications both in the response to transplantation, and in the development of auto-immune disease. The small intestinal connective tissue in contrast, expressed Ii more concordantly with its class II expression.
Antigenically secluded organs such as the heart, in common with most other organs, possess macrophage-like cells in the connective tissue which are intensely class II positive and highly mobile. These have been termed the interstitial dendritic cells, and are presumed to have potent antigen presenting capacity. The finding which most surprised us was the absence of the invariant chain in interstitial dendritic cells. This was surprising because it seems inconsistent with the presumed function of interstitial dendritic cells as sentinel antigen presenting cells (APCs) in the connective tissues. What are the consequences for antigen presentation of the absence of invariant chain, and why does the interstitial dendritic cell adopt this apparently odd phenotype? Without invariant chain, one would expect the class II molecules of interstitial dendritic cells to have a higher representation of cytosolic self peptides. One might also expect a lower cell surface expression of class II. However, given the intense staining for class II of the complex dendritic processes of interstitial dendritic cells (Hart et al. 1981b), it would seem that the absence of invariant chain has not influenced cell surface expression of MHC class II. Strong cell surface expression of class II can occur in the absence of invariant chain (Miller et al. 1986).

The mechanisms of self tolerance, and a rationalisation of the conflicting requirements of tolerance to self and immunity to external antigens, are only beginning to be understood. Matzinger, in a wide-ranging and detailed theoretical analysis (Matzinger. 1994), has proposed that T cell tolerance to peripheral-specific
self antigens is mediated either by parenchymal cells (which lack costimulatory ligands) or by interstitial dendritic cells (if these cells pass through a stage lacking costimulatory ligands). Much of her discussion on this point involves presentation for tolerance by parenchymal cells. However, we believe that our data, and some additional theoretical considerations, strongly favour the second hypothesis, i.e. a key role for the interstitial dendritic cell in peripheral T cell tolerance.

Clearly, the presentation of self antigens (whether self proteins originating in the dendritic cell itself or exogenous self proteins acquired by the dendritic cell) by a fully professional APC carries substantial risks of autoimmunity. T cell activation by the dendritic cell’s own self proteins could precipitate a catastrophic generalised immune stimulation, while activation towards peripheral specific antigens risks organ-specific autoimmunity. It is well known that tolerance to antigens present on dendritic cells occurs by clonal deletion in the thymus medulla, as discussed by Matzinger (Matzinger 1994). Such tolerance involves the virgin T lymphocyte pool. The peripheral pool consists to a large degree of memory T cells (Swain et al. 1996). These memory T cells have their adhesive molecules substantially upregulated (Sanders et al. 1988). It would seem wise to re-impose self tolerance in the periphery, since otherwise there would be a risk of autoimmunity from T cells responding to foreign peptides cross-reactive with self, and with a lower threshold for anti-self reactivity than the original virgin T cell. A phase where the invariant chain is not expressed might be the most effective way to ensure tolerance induction to a broader range of dendritic cell self peptides (including cytosolic peptides) than would otherwise be the case.
There is now evidence that freshly isolated LC (Austyn.1996) and interstitial dendritic cells (Austyn et al.1994) are poor T cell stimulators and that exposure to cytokines induces costimulatory ligands on dendritic cells (Hart et al.1993; Sallusto et al.1994). These characteristics, aided perhaps by a phase where there is lack of invariant chain, are precisely the requirements for the interstitial dendritic cell to have as its main function the induction of profound T cell tolerance to self antigens. Presentation of foreign antigens for T cell stimulation would then occur on a background of self-tolerance, and only when danger (Matzinger.1994) appears.

It is a truism that APC cannot distinguish self from non-self. However, experience is a hard teacher, and evolution might have taught the dendritic cell to present self first and foreign second and thereby, in an operational sense, to distinguish self from non-self. The epithet “professional” has been applied to dendritic cells because of their powerful ability to stimulate unprimed T cells. However, their professionalism might rest mainly with their high capacity to present antigen (because of high levels of expression of class II MHC molecules) and with their ready access to an exclusive part of the body, the T cell areas of organised lymphatic tissue (e.g. paracortex of lymph node, peri-arteriolar sheath of spleen).

With regard to the renal tubular cell, the absence of invariant chain would make excellent sense if the class II expression on this cell is for tolerance induction. The kidney specific molecules of the renal tubular cell, when shed, will be taken up as exogenous proteins by APC and presented by their class II MHC antigens to CD4+ T cells. The expression of invariant chain in the renal tubular cell would actually inhibit the presentation of these kidney specific proteins by the class II molecules of
the renal tubular cells, and thereby diminish their effectiveness for inducing
tolerance in CD4+ T cells. The observation of little invariant chain expression on
these cells may reflect the physiological lack of antigenic stimulus normally
prevalent in these organs.

It is fairly well established now that a major role for the invariant chain is in
providing the ability for class I and class II MHC glycoproteins to discriminate
between endogenous and exogenous antigenic peptides. The further ability of
different cell types in different tissues to regulate the exogenous peptides that they
present has not been ascertained. The data we present suggests that the invariant
chain, by way of differential regulation from the class II antigens, may play a role in
making this possible.
SECTION C - CHAPTER FIVE

C.5 ATTEMPTS TO DEMONSTRATE INDIRECT T-CELL ALLORECOGNITION OF DONOR MHC PEPTIDES IN TRANSPLANT PATIENTS

C.5.1 INTRODUCTION

The definition of the physiological pathway of antigen presentation (Berzofsky et al. 1988) and the elucidation of the crystal structure of MHC molecules (Bjorkman et al. 1987) highlighted the unusual nature of T cell recognition of allogeneic MHC molecules. In the normal immune response to foreign antigen, the T cell recognises antigen derived peptide fragments in association with self MHC class II molecules. The notable exception to this rule occurs in transplantation, where recipient T cells recognise foreign MHC antigen as undegraded molecules on the surface of allogeneic cells. This has been termed direct allore cognition, and provides a strong stimulus for both proliferative and cytotoxic T cell responses to the graft cells.

Antigen presentation by the conventional pathway of self MHC restricted presentation of donor MHC peptide, termed indirect allore cognition, has recently started to be recognised as a component of the rejection response. Depletion of donor antigen presenting cells (APC) in the form of passenger leucocytes occurs naturally after transplantation of vascularised organs, leaving recipient APC’s to replace them. In this situation, indirect presentation could play a significant role, in the generation and persistence of the chronic rejection response.
There is now abundant \textit{in vitro} (Essaket et al. 1990; De Koster et al. 1989) and \textit{in vivo} (Benichou et al. 1992; Dalchau et al. 1992; Fangmann et al. 1992b; Sherwood et al. 1986) evidence that indirect T cell recognition both occurs and can play a significant role in the effector mechanisms of rejection. Recipient T cell proliferation to donor MHC class I peptides presented by recipient APC's has been demonstrated following kidney (Fangmann et al. 1992b) and skin (Benichou et al. 1992) allografting in rodents. Evidence that this response actually contributes to the effector mechanisms of rejection has been obtained in rodents by transfer of primed syngeneic APC's (Sherwood et al. 1986), by selective sensitisation of the indirect T cell recognition pathway by donor peptides (Fangmann et al. 1993) or denatured MHC molecules (Dalchau et al. 1992), and by the use of MHC "deficient" mice (Auchincloss H Jr et al. 1993).

Although there has been much speculation as to the possible significance of these findings for clinical transplantation, there has not been a report demonstrating indirect T cell recognition of donor antigens in transplant patients. We have therefore attempted to detect recipient T cell proliferation to donor HLA-A and HLA-B incompatible peptides in paediatric patients undergoing chronic rejection of heart/lung allografts, and in adult patients following 1 or more episodes of acute kidney graft rejection. We decided to use 15mer peptides derived from donor histo-incompatible MHC class I molecules in T cell stimulation assays, using peripheral blood mononuclear cells purified from the blood of clinical organ graft recipients. The peptides were chosen from the hypervariable region of the \(\alpha_1\) and \(\alpha_2\) domains, of the 3 commonest MHC class I alleles of the A and B loci, in the Caucasian population, yielding a total of 12 15mer peptides to choose from. The
alleles chosen were HLA A1, A2, A3 and HLA B7, B8, B27. The peptides chosen for each specific assay were according to the donor MHC type: if there was a donor/recipient mismatch at one (or more) of the above alleles the peptides corresponding to the donor MHC were used in the subsequent proliferation assay. If there was a match for another of the alleles, the corresponding peptide was then used as a negative control in the same assay. In other cases, peptides from an irrelevant allele were used as the negative control. As a further refinement, peptide sequences were chosen with amino acids in certain positions, that corresponded to motifs known to bind preferentially in the binding groove of the HLA-DR1 allele (Chicz et al. 1992b).

C.5.2 MATERIALS AND METHODS

C.5.2.1 Buffers and Solutions

Phosphate Buffered Saline (PBS)

0.01M phosphate buffer, 0.0027M KCl, 0137M NaCl, pH 7.4 at 25°C

Normal Saline

9g NaCl per litre in distilled water (0.9%w/v)

Bovine Serum Albumin

10% w/v was prepared in PBS. 30g bovine serum albumin fraction V (Miles Scientific, Slough, UK) was added to 300ml PBS. Once dissolved this was dialysed twice against 5 litres of saline and once against PBS at 4°C and stored in 10ml aliquots at -20°C.
Trypan Blue

A stock solution of 1% w/v in distilled water was diluted 1/10 in PBS prior to use. An equal volume was added to the cell suspension before counting on a haemocytometer.

C.5.2.2 Peptides:

A series of 15 mer peptides (Table 1) were synthesised by Cambridge Research Biochemicals (Cambridge, Great Britain). These corresponded to the hypervariable regions (Parham et al.1988) of the most common HLA class I alleles in Caucasian populations (Essaket et al.1990). The various subtypes, e.g. of HLA-A2, did not differ in the region of the peptide (Zemmour et al.1993). The peptides were supplied in powder form, dissolved in phosphate buffered saline using sonication to assist solution and stored at 1mg/ml in small aliquots at -40°C.

C.5.2.3 Lymphocyte proliferation assay:

10mls of whole blood was collected in heparinised tubes, diluted 1:3 with 0.9% NaCl and Lymphoprep (Nycomed Pharma, Oslo) lymphocyte isolation suspension layered onto the bottom. The solution was spun and the lymphocyte layer taken off, washed twice in Heps buffered RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 1% human AB serum and glutamine, 2-mercapto-ethanol, penicillin and streptomycin as below. The cells were then counted and re-suspended at a concentration of 2x10⁶ cells/ml in RPMI 1640 (Flow Laboratories,
Irvine, Scotland) supplemented with 5% human AB serum, 2mM glutamine, $5 \times 10^{-5}$ M 2-mercapto-ethanol, 100 units/ml penicillin and 100µg/ml streptomycin. $2 \times 10^5$ cells were incubated in round-bottomed, 96 well tissue culture plates (Sterilin, Feltham, England) in 200µl volumes of the above culture medium. Cultures were stimulated in triplicate with the appropriate peptides as described above at a concentration of 40µg/ml, or with Concanavalin A (Con A) (Pharmacia, Milton Keynes, U.K.) at 10µg/ml as a positive control. Incubations were at 37°C in 5% CO$_2$ in air for 4 days. 24 hours before harvesting, 1µCi of $^3$H thymidine in 20µl of culture medium was added to each well. Cell-bound radioactivity was measured by placing dried filters in Optiscint Hisafe scintillation fluid (LKB, Milton Keynes U.K.) and counting in a LKB Rackbeta II liquid scintillation counter (LKB, Bromma, Sweden).

C.5.2.4 Patients

Three paediatric patients undergoing chronic rejection of heart/lung transplants (Cardiothoracic Transplant Unit, Great Ormond Street Hospital for Children Trust, London), and 12 adult patients after one or more episodes of acute rejection of kidney transplants (The Royal London Hospital) were studied (Table 2). Blood was taken from the paediatric patients at the time of bronchoscopy for diagnostic biopsy for slowly deteriorating lung function suggestive of chronic rejection (obliterative bronchiolitis). These patients were on baseline immunosuppression of cyclosporin, azathioprine and (except for patient 1) steroids.
Adult renal transplant recipients were recruited from The Royal London Hospital, Whitechapel (10 male, 2 female patients, mean age 38.9 years). All fulfilled clinical criteria for chronic rejection, and blood samples were taken at elective follow-up clinics. The diagnosis of acute cellular or vascular rejection had been confirmed by biopsy in all cases using the Banff criteria (Solez et al. 1993) (Table 2).

The 12 adult patients were on baseline immunosuppression of cyclosporin, azathioprine and prednisolone. These patients had had 1 or 2 preceding rejection episodes, and the blood for analysis was taken between 1 and 11 months after the last rejection episode.

C.5.3 RESULTS

Our results show that there was no detectable proliferation of lymphocytes when stimulated with the appropriate peptides. The counts ranged from 139cpm to 2686cpm for the 'stimulatory' peptides, and from 81cpm to 1136cpm for the negative control peptides. Con A stimulation of lymphocytes resulted in proliferation ranging from 4120cpm to 108200cpm. The peptide sequences, derived from the cDNA sequence, are given (in the one-letter code) in table 1. The patient details and corresponding data are shown in table 2. A typical proliferation result is given in Figure 1.
### TABLE 1: HLA peptides used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>HLA allele</th>
<th>Peptide</th>
<th>Amino acid position</th>
<th>Region of HLA molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>A1</td>
<td>PDGRFLRGYRQDAYD</td>
<td>105-120</td>
<td>β sheet</td>
</tr>
<tr>
<td>P2</td>
<td>A1</td>
<td>YWDLQTRNVKAQSQT</td>
<td>59-74</td>
<td>α helix</td>
</tr>
<tr>
<td>P3</td>
<td>A2</td>
<td>SDWRFLRGYHQYAYD</td>
<td>105-120</td>
<td>β sheet</td>
</tr>
<tr>
<td>P4</td>
<td>A2</td>
<td>AAQTTHKWEAAHVA</td>
<td>139-154</td>
<td>α helix</td>
</tr>
<tr>
<td>P5</td>
<td>A3</td>
<td>SDGRFLRGYRQDAYD</td>
<td>105-120</td>
<td>β sheet</td>
</tr>
<tr>
<td>P6</td>
<td>A3</td>
<td>SQTDRLGTRGYY</td>
<td>71-86</td>
<td>α helix</td>
</tr>
<tr>
<td>P7</td>
<td>B7</td>
<td>YWDRNTQIYKAQAQT</td>
<td>59-74</td>
<td>α helix</td>
</tr>
<tr>
<td>P8</td>
<td>B7</td>
<td>YLEGECVEWLRRYLE</td>
<td>159-174</td>
<td>α helix</td>
</tr>
<tr>
<td>P9</td>
<td>B8</td>
<td>YWDRNTQIKTNTQT</td>
<td>59-74</td>
<td>α helix</td>
</tr>
<tr>
<td>P10</td>
<td>B8</td>
<td>SQTDRESLRLRGGY</td>
<td>71-86</td>
<td>α helix</td>
</tr>
<tr>
<td>P11</td>
<td>B44</td>
<td>YWDRETQISKTNTQT</td>
<td>59-74</td>
<td>α helix</td>
</tr>
<tr>
<td>P12</td>
<td>B44</td>
<td>TQISKTNTQTYRENL</td>
<td>64-79</td>
<td>α helix</td>
</tr>
</tbody>
</table>
TABLE 2: Summary of patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Graft Received</th>
<th>Donor HLA type(^{(a)})</th>
<th>Recipient HLA type(^{(b)})</th>
<th>Time post-Transplant</th>
<th>Number of rejection episodes</th>
<th>Time from last rejection (months)(^{(c)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heart/Lung</td>
<td>A1,A2,B8,B39</td>
<td>A1,B8,B44</td>
<td>31 months</td>
<td>(chronic)</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>Heart/Lung</td>
<td>A2,A28,B8,B44</td>
<td>A1,B8,B44</td>
<td>41 months</td>
<td>(chronic)</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>Heart/Lung</td>
<td>A2,A31,B35,B49</td>
<td>A24,A19,B17,B21</td>
<td>21 months</td>
<td>(chronic)</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>Kidney</td>
<td>A2,B35,B44</td>
<td>A2,A3,B35,B65</td>
<td>12 months</td>
<td>1</td>
<td>11 (CR)</td>
</tr>
<tr>
<td>5</td>
<td>Kidney</td>
<td>A2,A32,B7,B35</td>
<td>A1,A32,B7,B44</td>
<td>10 months</td>
<td>2</td>
<td>9 (CR)</td>
</tr>
<tr>
<td>6</td>
<td>Kidney</td>
<td>A2,B8,B21</td>
<td>A1,A3,B7,B8</td>
<td>9 months</td>
<td>2</td>
<td>8 (VR)</td>
</tr>
<tr>
<td>7</td>
<td>Kidney</td>
<td>A1,A2,B8,B44</td>
<td>A1,A2,B18,B71</td>
<td>10 months</td>
<td>2</td>
<td>8 (VR)</td>
</tr>
<tr>
<td>8</td>
<td>Kidney</td>
<td>A1,A3,B18,B35</td>
<td>A3,A32,B14,B35</td>
<td>3 months</td>
<td>1</td>
<td>2 (CR)</td>
</tr>
<tr>
<td>9</td>
<td>Kidney</td>
<td>A1,A68,B8,B60</td>
<td>A2,A68,B8,B51</td>
<td>8 months</td>
<td>2</td>
<td>6 (CR\VR)</td>
</tr>
<tr>
<td>10</td>
<td>Kidney</td>
<td>A2,A3,B35</td>
<td>A1,A25,B8,B35</td>
<td>12 months</td>
<td>1</td>
<td>11 (CR)</td>
</tr>
<tr>
<td>11</td>
<td>Kidney</td>
<td>A3,A29,B7,B44</td>
<td>A3,A29,B44,B47</td>
<td>9 months</td>
<td>2</td>
<td>4 (VR)</td>
</tr>
<tr>
<td>12</td>
<td>Kidney</td>
<td>A2,A11,B8,B27</td>
<td>A2,B27,B44</td>
<td>2 months</td>
<td>1</td>
<td>1 (VR)</td>
</tr>
<tr>
<td>13</td>
<td>Kidney</td>
<td>A3,A68,B51,B44</td>
<td>A1,A24,B35,B71</td>
<td>10 months</td>
<td>1</td>
<td>7 (VR)</td>
</tr>
<tr>
<td>14</td>
<td>Kidney</td>
<td>A3,A26,B7,B45</td>
<td>A26,B27,B45</td>
<td>10 months</td>
<td>1</td>
<td>9 (CR)</td>
</tr>
<tr>
<td>15</td>
<td>Kidney</td>
<td>A2,A32,B7,B35</td>
<td>A3,B7</td>
<td>10 months</td>
<td>1</td>
<td>9 (CR\VR)</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Bold allele indicates allele used for allogeneic peptide
\(^{(b)}\) Allele in italics indicates allele used for syngeneic control peptide

Where there was no synthetic peptide corresponding to a syngeneic allele (patients 3, 14) irrelevant HLA peptides were chosen
\(^{(c)}\) The histological evaluation of the biopsy at the last rejection episode is given as cellular rejection (CR) or vascular rejection (VR) (Solez et al. 1993)
Table 3: Further details of rejection episodes from kidney patients

<table>
<thead>
<tr>
<th>Kidney Patient number</th>
<th>Date of most recent rejection</th>
<th>Date of blood sample (months from latest rejection)</th>
<th>Dates of previous rejections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21/12/92(CR)</td>
<td>8/12/93 (12)</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>8/3/93(CR)</td>
<td>8/12/93 (9)</td>
<td>19/2/93(CR)</td>
</tr>
<tr>
<td>3</td>
<td>23/4/93(VR)</td>
<td>8/12/93 (8)</td>
<td>8/3/93(CR), 17/3/93(CR)</td>
</tr>
<tr>
<td>4</td>
<td>24/4/93(VR)</td>
<td>15/12/93 (8)</td>
<td>16/3/93(CR/VR)</td>
</tr>
<tr>
<td>5</td>
<td>11/10/93(CR)</td>
<td>15/12/93 (2)</td>
<td>none</td>
</tr>
<tr>
<td>6</td>
<td>28/6/93(CR/VR)</td>
<td>22/12/93 (6)</td>
<td>19/4/93(CR/VR)</td>
</tr>
<tr>
<td>7</td>
<td>30/12/92(CR)</td>
<td>22/12/93 (12)</td>
<td>none</td>
</tr>
<tr>
<td>8</td>
<td>5/8/93(VR)</td>
<td>29/12/93 (4)</td>
<td>16/7/93(CR)</td>
</tr>
<tr>
<td>9</td>
<td>15/11/93(CR/VR)</td>
<td>29/12/93 (1)</td>
<td>none</td>
</tr>
<tr>
<td>10</td>
<td>17/5/93(VR)</td>
<td>29/12/93 (7)</td>
<td>none</td>
</tr>
<tr>
<td>11</td>
<td>13/2/93(CR)</td>
<td>15/12/93 (10)</td>
<td>none</td>
</tr>
<tr>
<td>12</td>
<td>22/2/93(CR/VR)</td>
<td>29/12/93 (10)</td>
<td>none</td>
</tr>
</tbody>
</table>

The histological evaluation of the biopsy at the last rejection episode is given as cellular rejection (CR) or vascular rejection (VR) (Solez et al.1993)
Figure 1: Typical T-cell proliferation assay - PBL from patient number 11 (Table 2) were cultured with syngeneic (HLA-A3) peptides 5 and 6 (Table 1), donor specific (HLA-B7) peptides 7 and 8 (Table 1), medium alone (med) or PHA. Cultures were harvested at day 4. Counts per minute indicate $^3$H thymidine incorporation during final 24 hours of culture. Means of triplicates are given. The standard error of the mean for PHA was ± 893 cpm. The range of cpm for the other 5 reactions was 254 to 1,117.
MHC class I rather than MHC class II peptides were chosen, as class I molecules are more abundant than class II in the commonly transplanted organs (Daar et al. 1984b; Daar et al. 1984a) and quantity of antigen (rather than the cell type on which it is present) is an important parameter for indirect T-cell recognition.

The reasons for the lack of stimulation of rejecting T-cells by peptides in vitro may be explained in a number of different ways. Firstly, it is conceivable that indirect recognition does not play a part in human transplant rejection, either overall or in the specific subset of patients we selected. The patients were chosen at the stage we thought it most likely that they would have their rejection episodes mediated by this pathway. Also, indirect recognition is well established in the animal model, and there is circumstantial evidence that the appropriate T cells are in place in the clinical situation. Secondly, the peptides chosen for this experiment may not be those being processed and presented by host APC’s. Previous studies in this laboratory provided evidence that this can be the case, wherein three class I peptides were chosen, 1 from the α helical region of the α₁ domain, 1 from the β sheet of the α₂ domain and 1 from the α helical region of the α₂ domain of the molecule, and used in the analogous experiment. T cell proliferation was seen with two of the three peptides used. We chose 12 peptides, from the α and β domains of the hypervariable region of MHC class I (2 peptides from each donor class I molecule), and the work in rodents suggests that synthetic peptides from these hypervariable MHC regions can frequently stimulate T cells in vitro (Benham et al. 1994; Benichou et al. 1992; Fangmann et al. 1992b).
Thirdly it may be that sensitised cells are too compartmentalised, and are not seen in sufficient numbers in the periphery to be able to elicit a proliferative response. Certainly, one major difference between this experiment and the rat study is that the stimulated cells in the rat assay were spleen cells, from culled animals, which are more likely to consist of a population of APC's and activated CD4 cells than peripheral blood. Fourthly, it might be difficult to detect indirect recognition in immunosuppressed patients. In the rodent, T-cells were harvested from non-immunosuppressed, primed recipients.

Next, the timing of the blood samples in relation to the rejection episode might not have been optimal. In the rodent, T cells were harvested after several days of an established rejection response. In the clinical setting, anti-rejection therapy is initiated early in the rejection response, which makes timing of the sample problematic. Finally, there may well be T cell sensitisation present in the periphery, but at too low a level to be detected by this assay.

T cells in vitro respond to allogeneic MHC molecules far stronger than to nominal foreign antigens. Early, acute graft rejection is primarily mediated by this 'direct' recognition. However these same T cells do not appear to induce late rejection of grafts in the animal model. This leaves a role for indirect presentation, particularly once donor APC's have migrated out of the graft. The possibility that indirect allorecognition might be important in transplantation was suggested by Butcher (Butcher et al.1982) and by Lechler (Lechler et al.1982) over 10 years ago. Benichou showed that T cells from allografted mice proliferated to synthetic
peptides derived from polymorphic regions of the donor class II molecules, presented by host APC's (Benichou et al.1992). Work in our laboratory has shown, additionally, that immunisation of rats with isolated, denatured chains of allogeneic MHC class I and class II molecules, primes indirect recognition, and results in accelerated second set allograft rejection, and also in T helper priming for antibody responses to the graft (Dalchau et al.1992).

Indirect presentation in clinical transplantation is practically more difficult to confirm. Suggestively, human CD4 cell lines have been described that recognise peptides derived from HLA class I molecules. Also, there is evidence that alloreactive T cells specific for human MHC class I antigens are self-restricted (BreurVriesendorp et al.1993). Our group has demonstrated that T cells responsive to selected donor MHC peptides are present in the graft rejecting animal, and can be shown to proliferate in vitro. Thus it would be logical to attempt to extend this experiment to human transplantation, in a search for a definitive demonstration of the indirect pathway in the rejection response, bearing in mind the logistical difficulties described above. The fact that this particular experiment was inconclusive does not preclude the role of the indirect pathway in clinical transplant rejection, but suggests that more complicated protocols will be necessary to detect indirect T-cell recognition in immunosuppressed transplant recipients. For example, if the opportunity ever presents itself, lymphocytes could be harvested from the graft or the spleen during a rejection episode. Whole donor MHC molecules, rather than synthetic peptides, might give a greater range of potential donor peptides. It might also be necessary to use assay techniques more sensitive than $^3$H thymidine incorporation. In any case, these additional complexities are likely to make it more
difficult to establish a role for indirect recognition in clinical transplantation, e.g. by correlating its presence with particular clinical outcomes such as the presence of chronic rejection.
SECTION D - DISCUSSION

The recognition of a transplanted organ by the physiological pathway of T-cell recognition, involves the breakdown of components of the graft into antigenic peptides, the transport of these peptides through the antigen presenting cell of the host, and presentation of the peptide on the cell surface in association with MHC class II molecules, for recognition by CD4 T-cells.

The importance of this mode of transplant recognition, in both acute and chronic forms, has recently begun to be appreciated. We have begun to look at the pathways involved in this process, with a view to a greater understanding of its overall role in allograft rejection.

One way in which we hope to understand MHC class II function, is by manipulating the control of its expression on specific cells pertinent to antigen presentation. The vascular endothelium is a permanent constituent of an organ graft that may possess antigen presentation capacity via its expression of MHC class II molecules. Class II molecules are not expressed on the vascular endothelium of rodents, whereas they are expressed on normal human vascular endothelium. The continuing presence of class II molecules on the vascular endothelium of a human organ graft, may, via the direct recognition pathway of presenting donor peptides to recipient T-cells, constitute a permanent source of immune stimulation, leading to chronic rejection. We surmise that this is one reason for the difference in rodents, wherein chronic rejection is not seen, and short term immunosuppression is adequate for long-term graft acceptance. To test this
hypothesis, we propose to induce class II expression on the vascular endothelium of the rat, and herein describe the first steps towards this aim.

In 1993, a new transactivating factor that controls expression of all class II isotypes, was cloned using cells from patients with MHC class II deficiency (Steimle et al.1993). This has been shown to have a specific action in binding to proteins that bind to the promoter region of the class II gene, and thereby being a necessary component in switching on the class II gene. The rat CIITA gene could thus be used in a transgenic experiment, in which a promoter specific for a molecule expressed constitutively and exclusively on vascular endothelium (e.g. the ICAM-2 promoter (Xu et al.1992)) would be attached to the CIITA gene, as the transgene construct. In this way it would be possible to express MHC class II constitutively on the vascular endothelium of long-term rat allografts.

The gene for CIITA has not to date been identified in any other species, and so the first step would be to clone the gene for rat CIITA. Our initial strategy to achieve this involved using a portion of the human CIITA cDNA as a radiolabelled probe to hybridise with DNA from a rat spleen library. This strategy, perhaps surprisingly, proved unsuccessful, probably due to either screening an insufficient number of plaques, or to using sub-optimal hybridisation conditions. At this point we decided that this hurdle would be best overcome by having a specific rat probe, which we might obtain by PCR of rat cDNA using primers from the human CIITA sequence. This approach would have the additional benefit of providing sequence data from a fragment of the rat gene which could be used for the design of antisense oligonucleotides. These oligonucleotides would then be used for in vitro experiments to switch off class II expression in various cell systems.
After experimenting with various primers, and reaction conditions, a PCR product was obtained which yielded a 433bp fragment of rat CIITA with 78.6% homology to the human gene. This has given us the basis with which to isolate the full length gene, and set up the experimental models envisaged.

The next stage of this experiment, as mentioned, is the design and use of antisense oligos to block gene expression. The techniques involved in antisense experiments are rapidly evolving, to the extent that patient trials are now underway in clinical oncology. The main use of antisense oligos to date has been to inhibit the expression of oncogenes or viral genes. The experiments we have performed have assessed the ability of these agents to suppress the expression of cytokine or co-stimulatory molecule genes in vitro, with a view to applying the experience gained, to suppress the CIITA gene. The end points used have been the measurement of PBMC proliferation, and an assay of IL-2 activity. It transpires that there is both a non-specific effect of oligos on cell proliferation, as well as a specific reductive effect. The specific effect can be considerably enhanced by the use of cell permeabilisation (using lipofectamine), which enables a substantial reduction in the dose of oligonucleotide, and thereby minimises the toxicity. The assay of IL-2 activity adds evidence that this is a gene specific effect. This experiment both illustrates that this is an alternative to using monoclonal antibodies in inhibiting cytokines in transplant rejection, and also that we could employ similar means to target the CIITA gene in due course.

The importance of recognising patients with MHC class II deficiency has been dramatically illustrated by the use of cells from these patients to identify hitherto unknown regulatory factors that exert overall control on MHC class II expression.
As well as CIITA, which controls the binding of a number of regulatory proteins to the class II promoter region, the gene for one of these proteins, RFX5 has also been identified from a subset of these patients (Reith et al. 1988). There are still two groups of patients in whom the molecular defect has not been identified, and we report here a further group of patients with MHC class II deficiency, originating from ethnic backgrounds not previously recognised as presenting with this condition. The ethnic bias reported in different complementation groups has been quite marked, indicating that our patients may indeed represent new complementation groups. We have performed immunohistology studies on fibroblast cell lines from these patients, and found that the invariant chain was not expressed.

Ii is a molecule important in antigen presentation in that it influences the repertoire of peptides presented by the class II molecule. We have demonstrated that Ii is not co-ordinately expressed with MHC class II in normal rat tissues. However, our fibroblast immunohistology studies imply that the control of Ii expression is also under the influence of factor(s) controlling class II expression. This has recently been shown in the case of CIITA, which appears to control multiple genes concerned with antigen presentation, but there is no information to date concerning the influence of RFX5 on Ii expression. Our systematic study of Ii and MHC class II expression on normal and transplanted tissues sheds light on the way in which different cell types may choose the repertoire of peptides they present via the class II pathway. In particular we were surprised by the lack of Ii expression in the interstitial dendritic cell, and postulate that this is a mechanism by which the IDC may play a key role in peripheral T-cell tolerance. This hypothesis might also
provide an explanation for the long-standing observation that class II antigens are expressed constitutively on epithelial cells of the proximal convoluted tubules of the kidney. We show that these cells lack Ii expression, implying that they physiologically present endogenous self peptide, perhaps for the purpose of tolerance induction.

Finally, to complete the picture, we have looked at the role of the ‘indirect pathway’ in the clinical setting of organ transplantation. This has been in practice difficult to confirm, following several recent animal studies suggesting its importance in graft rejection. Based on one of our previous animal experiments, we have stimulated peripheral blood mononuclear cells from chronically rejecting patients with peptides from donor MHC molecules. For reasons that are likely to be related to the practical difficulties in obtaining appropriately primed T-cells from patients, T-cell proliferation was not achieved in our *in vitro* assay. The role of indirect recognition in clinical transplantation remains difficult to define. The data presented in this thesis sheds additional light on antigen presentation and its regulation, with particular emphasis on the control and function of the MHC class II molecule, and provides the groundwork for further studies.
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