MONOCLONAL ANTIBODIES TO T-CELL DEFINED HLA-DR4 SUBTYPES PEPTIDE-DEPENDENT, ANTIBODY-BINDING EPITOPES ON HLA-DR β CHAINS ASSOCIATED WITH RHEUMATOID ARTHRITIS

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MONOCLONAL ANTIBODIES TO T-CELL DEFINED HLA-DR4 SUBTYPES

Peptide-Dependent, Antibody-Binding Epitopes on HLA-DR β Chains Associated with Rheumatoid Arthritis

by

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ABSTRACT

Histocompatibility molecules, encoded by genes of the major histocompatibility complex (MHC), play a pivotal role in shaping the T-cell repertoire by trapping peptides via their peptide-binding sites and presenting these MHC-peptide complexes for T-cell recognition. Among these molecules, one serologically-defined set called HLA-DR4 comprises several DRB1 variants or T-cell defined subtypes, in which the β chains differ from each other by only a few residues. The crossreactivity of some alloreactive T-cell clones and alloantibodies with certain DR4 and non-DR4 molecules that carry a homologous β chain sequence (residues 67-86) suggest that similar sequences generate similar structures. Such an element or "shared epitope" has been predicted to be implicated in the development and pathogenesis of rheumatoid arthritis (RA).

The present study was based on the prediction that T-cell defined determinants on HLA-DR4 subtypes and the "shared epitope" could be distinguished by antibodies and that such antibodies would be useful for structural analysis of these epitopes. Thus, the principal objectives were to develop such monoclonal antibodies (mAbs), to analyze differences in the "shared epitopes" on DR molecules that are associated with RA and to investigate the composition of these epitopes.

Approximately seventy anti-DR4 mAbs were selected from thirty fusions

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	alanine
ATCC	American Type Culture Collection
B-PBL	pepipheral blood B cell
BCL	B cell line
BSA	bovine serum albumin
C	cysteine
CDC	complement dependent cytotoxicity
CELISA	cellular enzyme-linked immunoassay
CLL	chronic lymphatic leukemia
D	aspartic acid
DMEM	Dulbecco's Modification of Eagle's Medium
DTAF	dichlorotriazinyl amino fluorescein
E	glutamic acid
EBV	Epstein Barr virus
ER	endoplasmic reticulum
F	phenylalanine
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	glycine
GAM	goat anti-mouse
H	hisitidine
HAT	hypoxanthine, aminopterin, thymidine
HLA	human leucocyte antigen
HRP	horseradish peroxidase
HTC	homozygous typing cell
HVR	hypervariable region
I	isoleucine
IHW	international histocompatibility workshop
Inv	invariant chain
K	lysine
Kb	kilobase
L	leucine
mAb	monoclonal antibody
Mb	million base pairs
MCF	mean channel fluorescence
MF	mean fluorescence

MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
N	asparaginine
OD	optical density
P	proline
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PE	phycoerythrin
Q	glutamine
R	arginine
RA	rheumatoid arthritis
RR	relative risk
S	serine
SEM	standard error of the mean
T	threonine
tk	thymidine kinase
V	valine
W	tryptophan
X	statistical mean
Y	tyrosine

CHAPTER 1

HLA-DR: GENETICS AND EXPRESSION

1.0 INTRODUCTION

Histocompatibility molecules, called HLA antigens in humans, were discovered independently by Dausset, van Rood and Payne about thirty-five years ago. They found that sera from multiparous and multi-transfused patients could agglutinate white blood cells from certain individuals. Shortly thereafter, through the use of rather primitive serology and skin grafting techniques, it was discovered that the degree of identity for these "human leucocyte antigens" correlated with skin graft survival or rejection in related and unrelated individuals. Thus, it was established that HLA antigens are major transplantation antigens and that they are codominately expressed (Amos, 1967). The story of these fascinating discoveries and the scientists who contributed immensely to this field has been chronicled in "History of HLA:Ten Recollections", edited by Terasaki (1990).

The importance of HLA molecules as major transplantation antigens is, however, secondary to their primary function, which is to bind and display antigenic peptides to self-restricted T cells (Zingernagel and Doherty, 1974). Two types of HLA molecules, class I and class II, evolved as structurally-related molecules, but with somewhat divergent functions. Essentially, class I molecules present

endogenously-derived peptides to cytotoxic T cells that express CD8 molecules, while class II molecules present both endogenously and exogenously-derived peptides to the helper/inducer T cells that express CD4 molecules (Rammensee et al., 1993). Virtually all nucleated cells constitutively express class I molecules on the cell surface, but the expression of Class II molecules is normally restricted to the membranes of antigen-presenting cells such as B cells, dendritic cells and macrophages.

All HLA molecules have a similar superficial groove, which contains most of the polymorphic amino acid residues that differentiate one specificity from another. This groove acts as a receptor for binding a wide variety of peptides (Bjorkman et al., 1987; Sette and Grey, 1992; Brown et al., 1993). Insertion of peptide into the class I groove occurs during assembly in the endoplasmic reticulum (ER) and this step appears necessary for correct folding, transport and surface expression (reviewed by Monaco et al. 1990). Class II molecules, on the other hand, associate with another chain, called the invariant (Inv) chain, which prevents peptide binding to the class II groove during assembly in the ER and during transport to the endosomal vesicles. At this latter stage the Inv chain dissociates which allows the class II groove to bind peptide (reviewed by Cresswell, 1992).

T cells that interact with MHC-peptide complexes are selected during ontogeny in the thymus to be self-MHC restricted. Thus, the majority of mature T cells express T cell receptors (TCR) that are self-tolerant and that recognize foreign

peptides in the context of self-MHC (von Boehmer et al., 1989). A consequence of MHC-restriction is that allo-MHC molecules are also recognized as foreign, which has serious implications for organ transplantation. The most unfavourable outcome of bone marrow transplantation is graft versus host disease (GVHD), which can occur even when there are minuscule differences between the HLA specificities. Intriguingly, this disease is reminiscent of autoimmune diseases, many of which are associated with subsets of particular HLA specificities.

The main focus of this review will be HLA-DR, including the complexities of its gene organization, its numerous polymorphisms and details of the expressed proteins. Emphasis will be placed on the structural features that determine the specificity and function of DR molecules. In the second part of this review (Chapter 2), the focus will be on the association of certain HLA-DR molecules, notably, DR4, DR1, DR14, and DR10 with Rheumatoid Arthritis.

1.1 OVERVIEW OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

HLA molecules are encoded by multiple genes in the major histocompatibility complex (MHC) which is located on the short arm of chromosome 6. The MHC extends over four million base pairs (4Mb) and contains approximately 100 genes, of which many code for non-HLA molecules (Campbell and Trowsdale, 1993).

Conventionally, the MHC has been divided into three main regions: class II at the centromeric end, followed by class III, and class I at the telomeric end (Figure 1.1). The class II region occupies about 1 Mb of DNA and is usually divided into subregions: HLA-DP, -DQ and -DR, but there are several other related and unrelated genes present. The organization of genes in this region, and the products that they encode, are detailed in section 1.2.

The class III region occupies about 1Mb of DNA and contains roughly forty non-HLA genes. Several genes code for proteins that have immunologically-related functions, such as the complement genes (C4, C2 and BF), heat shock proteins (HSP 70) and tumour necrosis factors (TNFA and TNFB). However, there are numerous other genes interspersed throughout this region and which are not depicted in Figure 1.1. The function of these genes is mostly unknown (Campbell and Trowsdale, 1993).

The largest segment is the class I region, which comprises the final 2 Mb. It contains between thirty and forty genes, including those coding for the heavy chain (α) of class I molecules (HLA-A, C and B). These genes are highly polymorphic and together with the beta-2-microglobulin (β_2M) gene encode more than 100 serological specificities. Forty-one HLA-A, 18 HLA-C, and 61 HLA-B alleles have been identified at the DNA level (Bodmer et al., 1992). The gene for β_2M is non-polymorphic and is encoded on chromosome 17. Other functional HLA genes in this region, are HLA-E, -F, and -G (reviewed by Geraghty, 1992).

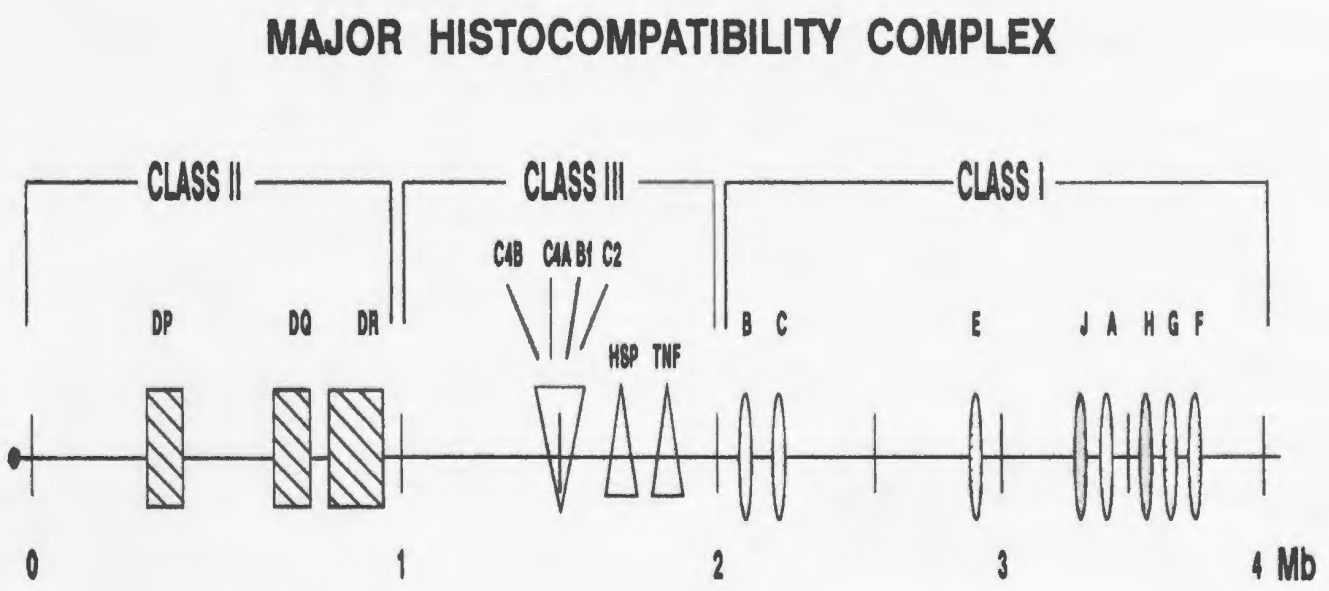

Figure 1.1. Schematic representation of the major histocompatibility complex, adapted from Campbell and Trowsdale, (1993). The centromere is indicated at the left. HLA-Class I: open symbols indicate polymorphic genes; dotted symbols, non-polymorphic genes; gray symbols indicate pseudogenes. Class II genes are depicted in Figure 1.2

HLA-E is poorly expressed on many different cells including T cells and eosinophils, while HLA-F and HLA-G are expressed during different stages of fetal development. Numerous HLA pseudogenes have also been mapped to this region.

1.2 ORGANIZATION OF HLA CLASS II GENES

The number of HLA genes and the order of the subregions (HLA-DP, -DQ and -DR) were originally mapped using a combination of cDNA and genomic cloning (reviewed by Korman et al., 1985; Trowsdale et al., 1985). Linking of these subregions and further identification of several new genes were accomplished by employment of new techniques and discoveries. These include the combination of rarely cutting restriction enzymes that cut unmethylated CpG-rich DNA sequences, which are associated with the 5' ends of genes (Lindsay & Bird, 1987; Bird, 1990), and pulse field gel electrophoresis (PFGE), (Hardy et al., 1986; Hanson et al., 1991). The use of the yeast artificial chromosome (YAC) cloning techniques also permitted the cloning of very large fragments of DNA (Ragoussis et al., 1991); this technique combined with PFGE was successfully used to determine the number of genes and the interlocus distances in different HLA-DR haplotypes (Ragoussis et al., 1992). A schematic diagram (Figure 1.2) of the class II region was adapted from the map published by Campbell and Trowsdale (1993).

HLA CLASS II REGION

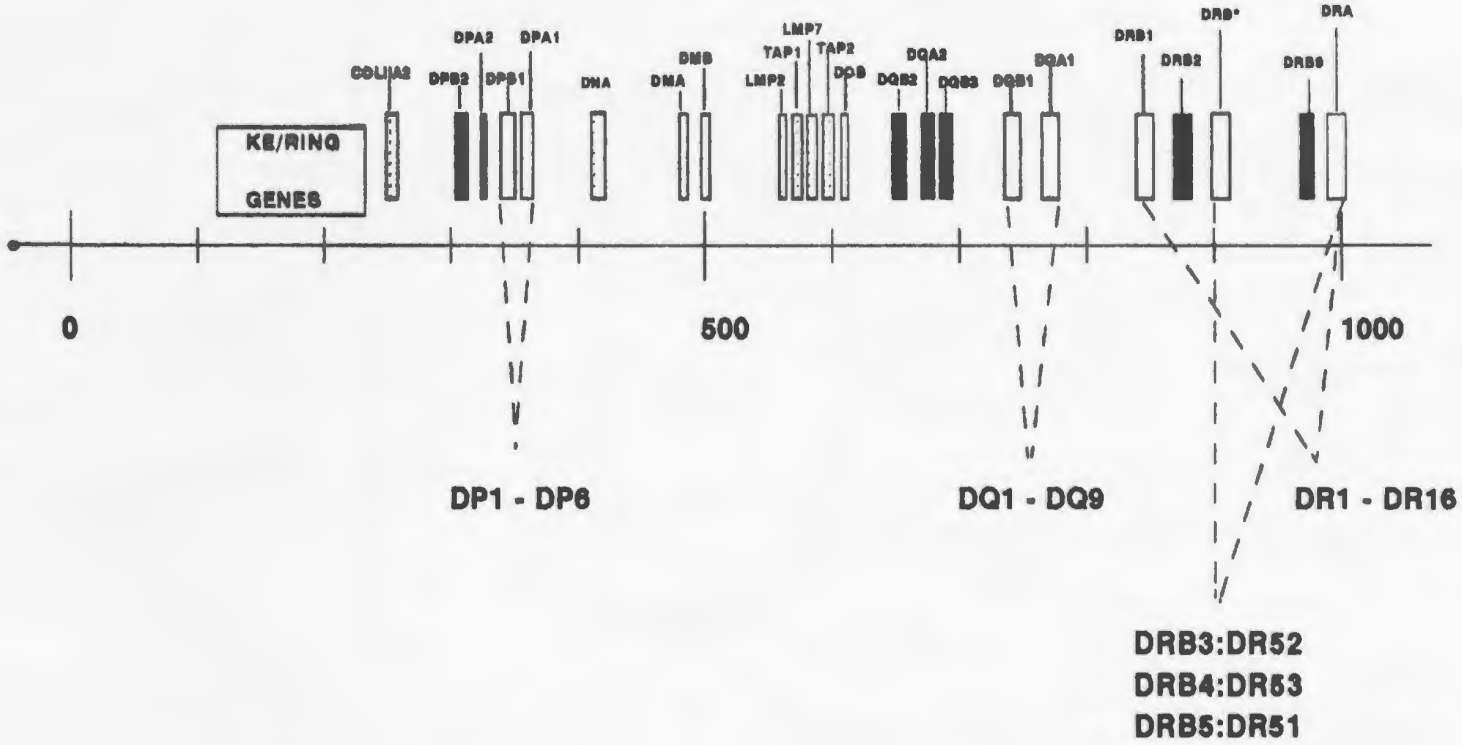

Figure 1.2 Organization of genes in the class II region, adapted from Campbell and Trowsdale, (1993). Open symbols indicate functional genes coding for class II specificities, DP1-6, DQ1-9,DR1-16, DR51, DR52, DR53; dotted symbols indicate poorly expressed HLA genes or non-HLA genes; black symbols indicate pseudogenes.

1.2.1A Non-HLA genes

Two large gene clusters containing several non-HLA genes have recently been mapped to this region. One cluster that maps between the DQ and DP subregions contains the TAP and LMP genes, which code for proteins that are involved in peptide loading of class I molecules (Monaco et al., 1990; Trowsdale et al., 1990; Deverson et al., 1990; Beck et al., 1992; Monaco, 1992; Powis et al. , 1993).

The TAP1 and TAP 2 genes belong to the ATP-binding cassette (ABC) superfamily, which code for a transporter molecule that ferries peptides from the cytoplasm to the lumen side of the endoplasmic reticulum (Trowsdale et al. , 1990). Binding of peptides to newly synthesized class I heavy chains precedes the association of β_2 microglobulin with the heavy chains and may be essential for the stable expression of class I molecules on the cell surface (Townsend et al., 1989; Spies et al., 1990). The LMP (low molecular weight polypeptide) genes are related to a structure with multiple proteolytic activities called multicatalytic proteinase complex or proteosome that is involved in the production of peptides for class I molecules (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991).

Several other genes are clustered centromeric to DP. The functions of these genes, for example the RING/ KE genes, are mostly unknown. Other genes such as the gene for collagen (type 11A2) are not immunologically relevant.

1.2.1B Classical HLA genes

The HLA-DR, -DQ and -DP genes extend over approximately 700 kilobases (Kb) of DNA in the class II region. Closely related genes (DNA, DMA, DMB, DOB), which lie between DPA1 and DQB2, are transcribed but poorly expressed on the cell membrane; their functions are essentially unknown. There are also several HLA-class II pseudogenes (DPA2, DPB2, DQB2, DQA2, DQB3, DRB2, DRB6, DRB7, DRB8 and DRB9), and the number of DRB pseudogenes depends on the haplotype of the individual (see section 1.2.2). The remaining HLA-DP, -DQ and -DR loci contain at least one A gene and one B gene that encode the $\alpha\beta$ dimer of a class II molecule or specificity. Many of these genes have several different allelic forms.

Following the 11th International Histocompatibility Workshop (IHW), the WHO nomenclature committee for factors of the HLA system completed and published a list of loci, genes, alleles and specificities (Bodmer et al., 1992). The list, at that time, included 8 DPA1 alleles and 36 DPB1 alleles, but only six different specificities (DP1 to DP6) were designated, as defined by primed lymphocyte testing. Similarly, although HLA-DQ genes are highly polymorphic with 14 DQA1 alleles and 19 DQB1 alleles described at the DNA level, only nine serological DQ specificities (DQ1 to DQ9) have been designated. Since the 11th IHW report, many new alleles, particularly for DPB1, have been described, but they will not be included here. Since the emphasis of this review is HLA-DR, the number of genes, alleles and specificities

for HLA-DR are considered in more detail in the following sections.

1.2.2 Genomic Organization of the HLA-DR Region

It was discovered several years ago, using Southern blotting and cosmid cloning techniques, that the number of functional DRB genes and DR pseudogenes differed according to the serologically-defined haplotype (Sorrentino et al., 1985; Rollini et al., 1985; Spies et al., 1985; Andersson et al., 1987). Confirmation of this has come from studies in which YAC cloning techniques and PFGE were used to map this region in several different haplotypes (Ragouissis et al. 1991; Ragouissis et al., 1992; Kendall et al., 1992). For example, the DR53 positive-haplotypes (DR4, DR7 and DR9) were found to contain 110 Kb of DNA more than the DR52 haplotypes (DR3, DR5 and DR6), (Kendall et al., 1992).

Five different groups of haplotypes (DR1, DR51, DR52, DR8 and DR53) were described by the HLA nomenclature committee (Bodmer et al.,1992). Grouping is based partly on sequence similarity in the 3' untranslated regions of the DRB genes and partly on linkage disequilibrium with other DR genes (Rollini et al., 1985; Spies et al., 1985; Mach et al., 1986; Gorski, 1989; Young et al., 1987; Erlich and Gyllensten, 1991). Recently, the members in these groups were also shown to have similar polymorphic regulatory sequences in the promoter regions of DRB genes

(Louis et al., 1993).

The number of DRB functional and pseudogenes are shown in Figure 1.3. The DRB1 gene is expressed in all haplotypes and together with DRA encodes the common serologically-defined specificities, DR1 to DR16, (Bodmer et al, 1992). No other known functional DRB genes are expressed in the haplotype groups DR1 (DR1, DR103 and DR10) or DR8 (Louis et al., 1993); however, the DR1 group contains the DRB6 and DRB9 pseudogenes. The DR51 group haplotypes (DR15 and DR16) also carry a DRB5 gene (DR51) and two pseudogenes DRB6 and DRB9; the DR52 group (DR3, DR11, DR12, DR13, and DR14) carries a DRB3 (DR52) and one pseudogene DRB2; the DR53 group haplotypes (DR4, DR7, and DR9) carry DRB4 (DR53) and three pseudogenes, (DRB7, DRB8 and DRB9).

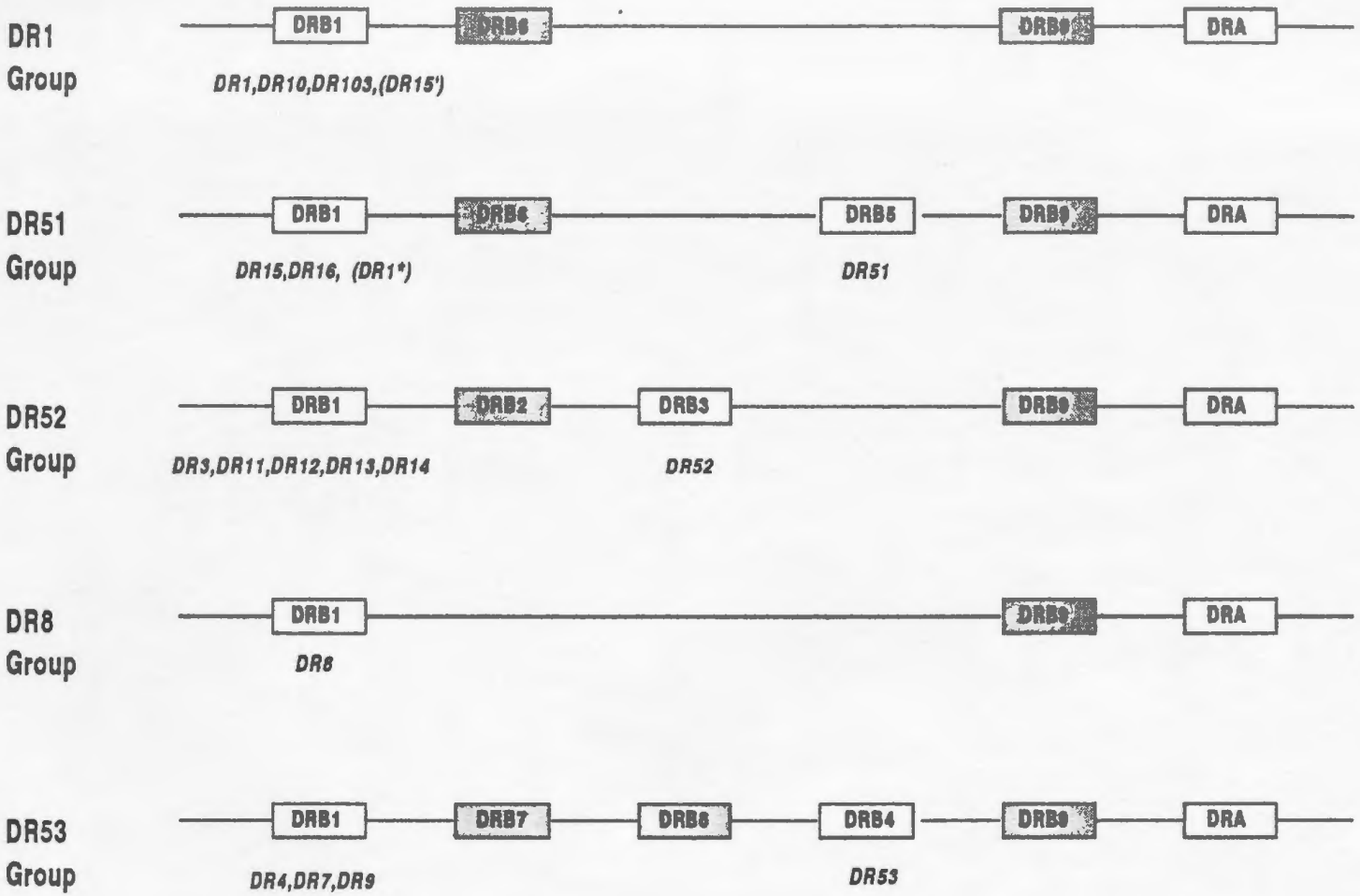

Figure 1.3 Schematic representation of the genomic organization of DR genes in the different haplotypes, adapted from Campbell and Trowsdale, 1993. Open boxes show the genes that code for the DR specificities; the gray goxes show the pseudogenes that are contained within each haplotype.

1.3 THE MOLECULAR STRUCTURE OF HLA-DR MOLECULES

1.3.1 Primary Structure

The primary structures of the alpha (α) and beta (β) chains of DR were partly determined by amino acid sequencing and more completely from nucleotide sequences derived from cloned cDNA and genomic DNA (reviewed in Shackleford et al., 1982). The arrangement of exons in DRA and DRB genes roughly corresponds to the number of domains identified in the polypeptide chains (Figure 1.4). The first exon codes for the signal sequences and the first two amino acids of the first domain. Expression of class II molecules is co-ordinately regulated by several promoter-like elements that are located 5' of the coding regions (Glimcher and Kara, 1993; Louis et al., 1993).

Both the α and β chains of DR as well as other class II molecules are Type 1 glycoproteins, which have the carboxyl termini located on the cytoplasmic side of the membrane, the hydrophobic segments spanning the membrane and the amino termini located on the external side of the membrane (Figure 1.4). The DR α chain is composed of 229 amino acids (15 internally, 23 transmembrane, and 191 externally) whereas the DRβ chain contains 237 residues (16 internally, 22 transmembrane and 199 externally), (Kaufman and Strominger,1979; Kratzin et al. 1981; Yang et al. 1982).

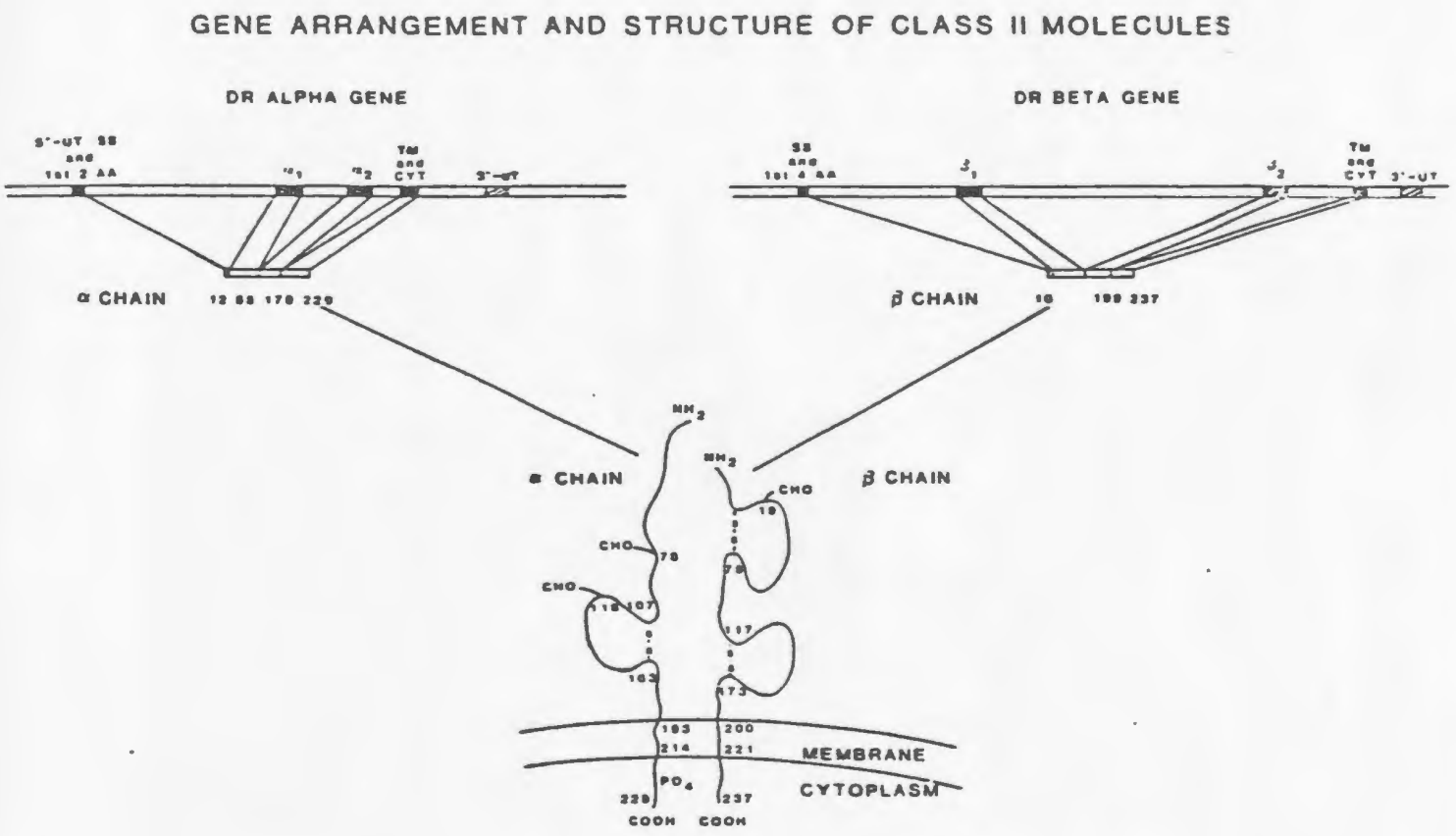

Figure 1.4 Schematic representation of the arrangement of exons that encode the domains of the DR alpha and beta chains. UT, untranslated; SS, signal sequence; TM, transmembrane; CYT, cytoplasmic. $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$, external domains of the alpha and beta chains respectively. (adapted from Stites et al., 1984)

The DRβ chain has one asparagine-linked oligosaccharide at position 19 while the DRα chain has two asparagine-linked oligosaccharides at positions 78 and 116 (Strominger 1980; Shackleford et al., 1982). Biochemical studies using tunicamycin to inhibit the formation of asparagine-linked sugars or endoglycosidases to remove sugars have shown that the carbohydrates contribute very little to the heterogeneity (Shackleford et al., 1983).

1.3.2 Secondary Structure

Both the alpha and beta chains have external domain-like structures (Figure 1.4). The α chain contains two domains α_1 (1-84) and α_2 (85-178), and is immunoglobulin-like due to a cysteine-linked disulphide bond. There are two immunoglobulin-like intrachain disulphide bonds in the β chain$_1$ (1-91) and β_2 (92-192). The α_1 and β_1 domains are distal and N-terminal while the α_2 and β_2 domains are proximal to the membrane (Kaufman and Strominger 1979; Kaufman et al., 1982; Korman et al.,1982).

Nucleotide sequencing of HLA-DR genes showed that the DR α chain is non-polymorphic and that the majority of the polymorphic residues are located in the first domain of the β chain (Long et al., 1983; Gustafsson et al., 1982; Cairns et al., 1985; Bell et al., 1985). It was apparent from Kabat and Wu-type variability plots that nearly all the polymorphic residues are clustered in three regions, called allelic hyper-

variable regions: HVR-I, codons 9-14; HVR-II, codons 25-38; HVR-III, codons 67-76 (Bell et al., 1987). These hypervariable regions are analogous to those that had earlier been described for murine I-A and I-E molecules (reviewed by Mengle-Gaw & McDevitt, 1985). These HVRs determine the individual serological and T-cell defined DR specificities. However, it was also apparent that there is extensive sharing of one or more of these HVR among different alleles. These shared regions or "patchwork patterns of allelic diversity" were predicted to have arisen by gene conversion events (Mengle-Gaw et al., 1984; Gorski and Mach, 1986; Erlich and Gyllensten, 1991).

1.3.3 Three-dimensional Structure

Resolution of the X-ray crystallographic structure of a class I (HLA-A2) molecule by Bjorkman et al., (1987) was a major step in clarifying the primary function of HLA molecules in immune recognition. Its most revealing feature was a shallow groove or cleft that was formed by the pairing of the α_1 and α_2 domains. This groove consisted of eight antiparallel beta-strands with an alpha-helical structure on each side. Nearly all of the known polymorphic amino acid residues in the HLA-A2 molecule were located on the floor or sides of the groove; remarkably, the groove contained bound peptide. From these observations, it was predicted that the side chains of residues, which point up from the floor or inwards from the alpha helix,

would bind peptide and that the side chains of those residues that point upwards and outwards from the alpha helix would bind the T cell receptor.

By aligning the homologous regions of class II molecules with those of class I molecules, Brown et al. (1988) constructed a hypothetical model with a peptide binding site that was similar to that of the HLA-A2 molecule. Recently, the same group published the three-dimensional (3-D) structure of a DR1 crystal, which is comparable to the class I structures and the Brown model (Brown et al., 1993). For example, the β_2 and α_2 domains of the class II molecules correspond to the α_3 domain and β_2M, respectively of class I. The class II β_1 and α_1 domains, like the α_2 and α_1 domains of class I, interact to form a similar peptide binding groove.

There are some dissimilarities between the 3-D structures of class I and class II molecules, particularly with respect to the peptide binding site. First, the grooves of human and mouse class I are closed at both ends and accommodate peptides of 8-10 residues with longer ones bulging in the middle (Jardetsky et al., 1991; Madden et al., 1991; Zhang et al., 1992). The class II groove is open at both ends and binds peptides that are considerably longer (15-24AA) and protrude from both ends (Rudensky et al., 1991; Chicz et al., 1992; Brown et al., 1993). While specific peptide-binding pockets in the class I groove have been readily identified from sequences of eluted peptides (Rotzschke et al., 1991; Falk et al., 1991; Hunt et al., 1992), only one clearly identifiable pocket has been discerned in the class II groove. This is a hydrophobic pocket located at one opening of the groove and includes the

cluster (β86, 89,90; α31,52). Other pockets are suggested by the clustering of particular polymorphic amino acids in the groove such as (β13,26,28,71,74,78), (β9,11,30; α66), (β37,38,58). Another major difference is that in all three DR1 crystals, two dimers were associated with each other ($dimers_2$) whereas none of the class I crystals were in this state.

1.4 BIOSYNTHESIS AND EXPRESSION OF HLA-DR MOLECULES

1.4.1 Assembly

Transcription of the class II genes is co-ordinately regulated by promoter sequences found 5' of the coding regions (reviewed by Glimcher and Kara, 1992). Synthesis of the α and β polypeptides occurs on the polysomes of the rough endoplasmic reticulum, after which they are normally assembled as isotypic DRαβ, DQαβ, or DPαβ molecules. Mismatched pairing of the isotypic chains can potentially occur. This was clearly demonstrated by DNA-mediated transfer experiments in mice in which cloned genes from $I\text{-}A_\beta^d$ and $I\text{-}E_\alpha^{a/d}$ were expressed as mixed molecules on the cell surface of transfected class II negative L cells (Germain and Quill, 1986). The experiments also showed that this pairing was determined by polymorphic sequences in the amino terminus of the I-Aβ chain. Recent studies have shown that such aberrant molecules are functional in that they are capable of antigen

presentation (Ruberti et al., 1992)

Trans-associated or hybrid DQ molecules have been detected in immunoprecipitates (Charron et al., 1984; Nepom et al., 1987). This phenomenon was also experimentally demonstrated by transfection and expression of haplotype mismatched DQA and B genes (Kwok et al., 1988); it was later shown by the same group that not all DQ β chains could associate with all DQα chains (Kwok et al., 1993). Again drawing on studies done earlier in the murine system, it appears that allelic and interallelic pairing and expression depend on the correct combination of polymorphic residues in the amino terminus of the chains (reviewed by Germain and Malissen, 1986; Braunstein et al., 1987).

1.4.2 Role of the Invariant Chain

Several years ago it was shown, by electrophoretic analysis of immunoprecipitates from biosynthetically labelled class II molecules, that the class II heterodimer is associated with another molecule, which is called the gamma or Invariant (Inv) chain (Jones et al., 1979). The initial experiments suggested that the Inv chain assisted in transporting the $\alpha\beta$ dimer from the ER through the various sub-cellular compartments and inserting it into the cell membrane (Kvist et al., 1982). This theory was challenged when it was clearly shown that transfected class II genes could be expressed in Inv negative fibroblasts that do not constitutively express class

II (Miller and Germain, 1986; Sekaly et al., 1986). Transfected class II molecules expressed on the Inv negative fibroblasts were found to have altered antibody-binding epitopes and/or peptide presentation capabilities (Petersen & Miller, 1990; Roche et al., 1990), thereby renewing interest in the function of the Inv chain.

The structure of Inv differs significantly from that of the $\alpha\beta$ subunits (reviewed by Cresswell, 1992). It is a type II glycoprotein, which has the amino terminal on the cytoplasmic side, a hydrophobic transmembrane piece and a lumenal carboxyl terminal. It is also heavily glycosylated and has several alternate forms, with the majority being 31-33Kd. Newly synthesized Inv forms a trimer and each member of the trimer can bind a class II heterodimer through a signal piece in its carboxyl lumenal region (Roche et al., 1991). This piece remains associated with class II during transport of the nonameric complex from the ER through the Golgi and trans-Golgi reticulum (Teyton et al., 1990). Here, the complexes are sorted to the endocytic route by the same targeting signal in the cytoplasmic tail of the Inv chain. In the acidic environment of the endosomes, processing of exogenous antigens occurs (Neefjies et al., 1990). Arrival of the complex into these compartments results in degradation of the Inv chain and binding of processed peptide to the class II groove. The peptide-loaded class II molecules are then transported to the cell surface by some unknown mechanism. As a further note to the chaperon-like role of Inv, Anderson and Miller (1992) recently showed that transportation and expression of class II molecules in Inv negative cells is considerably less efficient than in Inv

positive cells, and that such molecules are abnormally glycosylated and conformationally unstable.

1.4.3 Peptide Involvement in the Conformation of Class II Molecules

Several studies have established the importance of peptide binding to the groove in sustaining the overall structural features of the molecule (Sadegh-Nasseri and Germain 1991; Germain and Hendrix, 1991). Using chain-specific monoclonal antibodies to purify molecules from the different subcellular compartments, it was found that newly synthesized and assembled dimers were unstable and easily denatured, whereas molecules that had matured in the post Golgi reticulum and endosomal compartments were more stable and resistant to denaturation. Furthermore, class II molecules expressed in cells that have large deletions in the MHC region and that are known to be defective in antigen processing, were also conformationally unstable; such molecules had modified peptide-presentation capabilities (Mellins et al. 1990a; Riberty and Cresswell, 1992; Sette et al. 1992). The majority of class II molecules that were purified from antigen-processing cells contained a large piece of the Inv chain that had not dissociated, presumably, because there was a deficiency of processed peptide in the endosomal compartments (Sette et al., 1992). DeNagel and Pierce (1992) have further suggested that after dissociation of the Inv chain, other proteins belonging to the HSP70 family may be

required in order to facilitate peptide binding to the groove. They speculate that the HSP may do this by directly transporting the peptide to the groove or by transporting peptides from a different compartment to one containing the class II molecules.

1.4.3A Characteristics of peptides isolated from class II grooves

Most of the peptides, which have been eluted from murine and human class II molecules, are derived from membrane and secretory proteins (Rudensky et al., 1991, Chicz et al., 1992). For example, Chicz et al. (1993) found that about 85% of the peptides eluted from HLA-DR molecules were derived from endogenous proteins. This was unexpected since Class II molecules were predicted to bind primarily exogenously-derived peptides. Neefjies and Ploegh (1992) have suggested that these endogenously-synthesized proteins may gain access to the endosomal compartments by autophagy and/or translocation from the cytosol to lysosomes. Like exogenous antigens, they may then be processed into peptides that can bind to class II molecules.

Peptides isolated from class II molecules are, also, longer and more variable (12-25 mer) than peptides that have been eluted from class I molecules. They are also characterized by so-called "ragged ends" (Rudensky et al., 1991; Hunt et al., 1992; Chiez et al., 1993). This feature is due to the class II binding site being open at both ends (Brown et al., 1993), so that a typical 15-17mer peptide, depending on its chemical composition and the composition of the groove, might bind in different

forms. Only a core of 8-10 residues actually binds while the other residues overhang both ends of the groove.

Peptide-binding motifs were not readily apparent from comparison of the sequences of eluted peptides that were isolated from a variety of DR molecules; furthermore, most 15-17 mer peptides bound to all or most class II molecules (Rudensky et al. 1991; Chicz et al. 1992; Chicz et al., 1993). This supported the findings of previous studies, in which a high level of degenerate peptide binding by class II molecules was found; (Rothbard and Gefter, 1991; O'Sullivan et al. 1991). Recently, a large M13 peptide display library was generated to screen isolated DR molecules for peptide motifs that are associated with binding to DR (Hammer et al., 1992; Hammer et al., 1993). Peptide motifs, which include a broad range of binding with respect to a variety of DR molecules, were found. These had so-called promiscuous anchor residues at positions 1 and 4 and allele-specific residues at position 6. The amino and carboxyl termini of the peptides are known to be oriented with the amino and carboxyl termini of the peptide binding site (Brown et al., 1993). Therefore, Hammer et al., (1993) predicted that promiscuous anchor residues (anchor positions in the amino end of the peptide) bind to the non-polymorphic DR α positions (amino end of the groove), while allele-specific anchor residues, which are in the carboxyl end of the peptide, bind to a pocket created by the polymorphic residues in the β strands (carboxyl end of the groove). Similar peptide motifs for binding to HLA-DR4 molecules have also been described by Sette et al (1993).

1.5 HLA-DR SPECIFICITIES

The discovery of the mixed lymphocyte culture reaction (MLC) by Bain et al., (1964) and Bach et al., (1964) led to the identification of HLA-D and later to recognition of HLA-Dw determinants (Yunis and Amos, 1971). Similar specificities that were identified on B lymphocytes by alloantisera (van-Leeuwen et al., 1973; van Rood et al., 1975; van Rood et al., 1976) were named HLA-DR after the 7th IHW,1977 because they were "related" to the cellular-defined HLA-Dw determinants (see review by Winchester and Kunkel, 1979). The fact that Dw and DR are different determinants on the same molecule, was obscured for many years because several Dw subtypes can associate with a single serological specificity. For example, the HLA-DR4 specificity was shown to contain at least five different T-cell Dw subtypes (Groner et al., 1983; Reinsmoen et al., 1982). The relationship of DR-Dw was clarified by cDNA cloning and nucleotide sequencing (Cairns et al., 1985; Bell et al., 1985; Gregersen et al., 1986) Subsequently, DNA typing methods have been used to identify several new variants for most DR specificities (Kimura et al., 1992; Bodmer et al., 1992; Stastny and Kimura, 1992).

1.5.1 HLA-DR Nomenclature

The HLA nomenclature for serological specificities, splits and subtypes, was revised after the 1987 IHW in an effort to consolidate all the information obtained from serological, T-cell and DNA typing and sequencing (Dupont, 1989); the most recent update followed the 11th IHW (Bodmer et al., 1992). A partial list of DRB alleles, for which serologically-defined and/or cellular-defined specificities have been identified, is shown in Table 1.1; it should be noted, however, that several other functional alleles have been identified at the gene, but not at the product level. The following is an interpretation of the new nomenclature.

A serological specificity refers to a group of molecules that carries determinants common to all molecules within that specificity; it may be encoded by one (for example DR10) or several related alleles (for example DR4). Each of the listed alleles have been DNA-typed and sequenced before assigning the locus, allele and number. In the example DRB1*0102, DRB1* indicates that it is a functional allele encoded by DRB1; the first two numerals (01) indicate the specificity (DR1) and the second two numerals (02) indicate that it is the second allele in the DR1 specificity. Thus, the serologically-defined DR1 specificity consists of a DRβ1 chain, (encoded by either DRB1*0101, B1*0102 or B1*0103), which is associated with the invariant DRα chain. Within each DR group, there are T-cell defined specificities that are referred to as Dw subtypes. Each subtype is encoded by distinct DRB alleles;

Table 1.1 Common HLA-DR serological and T-cell defined specificities and the DRB genes that encode them.

Serological Specificities	T-cell-Defined Specificities	DRB1*	DRB5	DRB3	DRB4
DR1	Dw1	0101			
DR1	Dw20	0102			
DR103	Dw'BON'	0103			
DR10	-				
DR15 (2)	Dw2	1501	DR51 0101		
DR15 (2)	Dw12	1502	DR51 0102		
DR16 (2)	Dw21	1601	DR51 0201		
DR16 (2)	Dw22	1602	DR51 0202		
DR17 (3)	Dw3	0301		DR52	
DR17 (3)	Dw'RSH'	0302		DR52	
DR11 (5)	Dw5	11011/11012		DR52	
DR11 (5)	Dw'JVM'	1102		DR52	0101
DR11 (5)	Dw'FS'	1103		DR52	0201
DR12 (5)	Dw"DB6"	1201		DR52	0202
DR13 (6)	Dw18	1301		DR52	0301
DR13 (6)	Dw19	1302		DR52	
DR13 (6)	Dw'HAG'	1303		DR52	
DR14 (6)	Dw9	1401		DR52	
DR14 (6)	Dw16	1402		DR52	
DR8	Dw8.1	0801			
DR8	Dw8.2	08021/08022			
DR8	Dw8.3	08031/08032			

Table 1.1 Continued

Serological Specificities	T-cell-Defined Specificities	DRB1*	DRB5	DRB3	DRB4	
DR4	Dw4	0401			DR53	0101
DR4	Dw10	0402			DR53	0101
DR4	Dw13.1	0403			DR53	0101
DR4	Dw14.1	0404			DR53	0101
DR4	Dw15	0405			DR53	0101
DR4	Dw'KT2'	0406			DR53	0101
DR4	Dw13.2	0407			DR53	0101
DR4	Dw14.2	0408			DR53	0101
DR7	Dw17	0701			DR53	0101
DR7	Dw'DB1'	0702			DR53	0101
DR9	Dw23	09011/09012			DR53	0101

for example Dw1 is encoded by DRB1*0101. Since serological and T-cell typing methods were in use long before DNA sequencing and typing methods were available, the nomenclature for the Dw specificities does not necessarily match numerically with the DRB1* alleles.

1.5.2 Serological Determinants: Location and Composition

Elucidation of the primary and secondary structure of DR molecules revealed that the β_1 domain contained almost all of the polymorphic residues. Variability plots indicated that these polymorphic residues clustered in three regions of the first domain: HVR I, 9-13; HVR II, 25-38; HVR III, 67-74 **(see Section 1.3.2)**. When these residues are localized to the 3-D structure of the DR1 molecule, it can be seen that HVR I and HVR II are on the β-pleated sheets while HVR-III is located on the α helix. From analysis of the sequence data for the many different specificities that have been either serologically or T-cell defined, it is clear that many DR specificities do not have allele-specific amino acid residues (Figure 1.5). No antisera have been described which strictly correlate with a Dw specificity, but by using a combination of allosera with "short specificities" patterns were obtained that correlated with the product of a single DRB1 allele (Juji et al., 1992).

		AMINO ACID RESIDUES						
		HVR I	HVR II				HVR III	
SPECIFICITY	ALLELE	9-16	26-33	37-38	47	57-60	67-78	85-86
DR1 Dw1	B1*0101	WQLKFECH	LLERCIYN	SV	Y	DAEY	LLEQRRAAVDTY	VG
DR1 Dw20	B1*0102	WQLKFECH	LLERCIYN	SV	Y	DAEY	LLEQRRAAVDTY	AV
DR103 DwBON	B1*0103	WQLKFECH	LLERCIYN	SV	Y	DAEY	ILEDERAAVDTY	VG
DR4 Dw4	B1*0401	EQVKHECH	FLDRYFYH	YV	Y	DAEY	LLEQKRAAVDTY	VG
DR4 Dw10	B1*0402	EQVKHECH	FLDRYFYH	YV	Y	DAEY	ILEDERAAVDTY	VV
DR4 Dw13	B1*0403/7	EQVKHECH	FLDRYFYH	YV	Y	DAEY	LLEQRRAEVDTY	VV/G
DR4 Dw14	B1*0404/8	EQVKHECH	FLDRYFYH	YV	Y	DAEY	LLEQRRAAVDTY	VV/G
DR4 Dw15	B1*0405	EQVKHECH	FLDRYFYH	YV	Y	SAEY	LLEQRRAAVDTY	VG
DR4 DwKT	B1*0406	EQVKHECH	FLDRYFYH	SV	Y	DAEY	LLEQRRAEVDTY	VV
DR3 Dw3	B1*0301	EYSTSECH	YLDRYFHN	NV	F	DAEY	LLEQKRGRVDNY	VV
DR11 Dw5	B1*1101	EYSTSECH	FLDRYFYN	YV	F	DEEY	FLEDRRAAVDTY	VG
DR11 DwJVM	B1*1102	EYSTSECH	FLDRYFYN	YV	F	DEEY	ILEDERAAVDTY	VV
DR12 DwDB6	B1*1201	EYSTGECY	LLERHFHN	LL	F	VAES	ILEDRRAAVDTY	AV
DR13 Dw18	B1*1 301	EYSTSECH	FLDRYFHN	NV	F	DAEY	ILEDERAAVDTY	VV
DR13 Dw19	B1*1302	EYSTSECH	FLDRYFHN	NV	F	DAEY	ILEDERAAVDTY	VG
DR13 DwHAG	B1*1303	EYSTSECH	FLDRYFYN	YV	Y	SAEY	ILEDKRAAVDTY	VG
DR14 Dw9	B1*1401	EYSTSECH	FLDRYFHN	FV	Y	AAEH	LLERRRAEVDTY	VV
DR14 Dw16	B1*1402	EYSTSECH	FLERYFHN	NV	Y	DAEY	LLEQRRAAVDTY	VG
DR8 Dw8.1	B1*0801	EYSTGECY	FLDRYFYN	YV	Y	SAEY	FLEDRRALVDTY	VG
DR8 Dw8.2	B1*0802	EYSTGECY	FLDRYFYN	YV	Y	DAEY	FLEDRRALVDTY	VG
DR8 Dw8.3	B1*0803	EYSTGECY	FLDRYFYN	YV	Y	SAEY	ILEDRRALVDTY	VG
DR7 Dw7	B1*0701	EYGTYKCH	FLERLFYN	FV	Y	VAES	ILEDRRGQVDTV	VG
DR7 DwDB1	B1*0702	EYGTYKCH	FLERLFYN	FV	Y	VAES	ILEDRRGQVDTV	VG
DR9 Dw23	B1*0901	KQDKFECH	YLHRGIYN	NV	Y	VAES	FLERRRAEVDTV	VG
DR15 Dw2	B1*1501	WQPKRECH	FLDRYFYN	SV	F	DAEY	ILEQARAAVDTY	VV
DR15 Dw12	B1*1502	WQPKRECH	FLDRYFYN	SV	F	DAEY	ILEQARAAVDTY	VG
DR16 Dw21	B1*1601	WQPKRECH	FLDRYFYN	SV	Y	DAEY	FLEDRRAAVDTY	VG
DR16 Dw22	B1*1602	WQPKRECH	FLDRYFYN	SV	Y	DAEY	ILEDRRAAVDTY	VG
DR15 Dw2	B5*0101	QQDKYECH	FLHRDIYN	DL	Y	DAEY	FLEDRRAAVDTY	VG
DR15 Dw12	B5*0102	QQDKYECH	FLHRGIYN	NV	Y	DAEY	FLEDRRAAVDTY	VG
DR16 Dw21	B5*0201	QQDKYECH	FLHRGIYN	NV	Y	DAEY	ILEQARAAVDTY	AV
DR16 Dw22	B5*0202	QQDKYECH	FLHRGIYN	NV	Y	DAEY	ILEQARAAVDTY	AV
			β Sheet				α Sheet	

Figure 1.5 Polymorphic amino acid sequences of the β_1 domain of DR β chains which are associated with T-cell defined specificities.

1.5.2A Hypothetical mapping by analysis of sequence data

The location and composition of certain serological epitopes on HLA molecules can be predicted by comparing the antibody-binding patterns, which are obtained with homozygous HLA cell lines, to the available amino acid sequence data. Mouse cell transfectants expressing wild-type HLA genes have proven extremely valuable for localizing the antibody-binding epitope to the gene product (Klohe et al., 1988; Madrigal et al., 1989; Heyes et al., 1992; **CHAPTER 6**). By comparing antibody binding data from several sources to class II sequence data, epitopes for several well known mAbs have been predicted (Marsh and Bodmer, 1989; Marsh et al., 1992). For example, they have predicted that DR7-specific mAbs bind to either DRB1:11-14:GKYK, 25:Q, 30:L or 71-74:RRGG. Similarly, Gorski et al., (1990) putatively mapped a monoclonal antibody-binding epitope to a shared sequence (DRB1:13G, 16Y), which is only found on DR8 and DR12 molecules.

Extensive analyses were carried out during the 11th IHW using computer software to compare reaction patterns that had been obtained using several thousand HLA antisera against well-characterized B cell lines with the class II amino acid sequences (Clarke et al., 1992; Barbetti et al., 1992). They found that several DR specificities correlated with the presence of a single allele-specific residues on DR β chains, for example, DR1, DR3, DR4, DR7, DR9, DR10, DR11, DR12, and DR51. In addition, so-called "multi-specific" antisera or "broadly-reactive" mAbs could be mapped to a single residue or a short sequence found on several different DR β

chains. It is of interest that many of these single residues, which have been suggested as antibody-binding epitopes, are located on the floor of the peptide-binding site at positions that are involved in peptide binding (Brown et al., 1993). If this is correct, it seems unlikely that antibodies could interact directly with these residues as their side chains would be obscured by peptide and, thus, unavailable for antibody binding.

1.5.2B The analytical approach

One would predict that serological epitopes on DR molecules are similar to most other antibody-binding epitopes on native proteins. That is, they are formed by the combination of several amino acids that are linearly distant in the primary structure, but spatially-associated in the 3-D structure (Laver et al., 1990). X-Ray crystallographic analyses of different Fab-Ag complexes have revealed that the epitopes on lysozyme and neuraminidase occupy a large area comprising 15-17 amino acid that occur on different strands (Amit et al., 1986; Tulip et al., 1992). Such complexes have a high degree of topographic and chemical complementarity at their interacting surfaces (Maruzza and Poljak, 1993). Although a subset of the residues in the epitope contribute most of the binding energy, they are not usually arranged in a linear way. Thus, it seems unlikely that an epitope for an anti-HLA-DR antibody will be mapped to a single amino acid; it is likely, however, that an "allele-specific" residue may be critical for the overall structural integrity of an epitope.

Unequivocal mapping of an alloepitope would require the co-crystallization of a Fab fragment and its cognate antigen, but since this is not feasible, alternate methods have been employed. These involve the use of cells that express altered class II genes, such as mutants that have been immunoselected for loss of antibody-binding epitopes. Such mutants were first used for mapping epitopes on murine class II molecules (Glimcher et al., 1983; Beck et al 1984; Beck et al. 1991). Sequencing of the relevant genes from these cells identified the mutations that generated a structural alteration in the MHC molecule with subsequent loss of the binding epitope. For example, Beck et al, (1991) mapped several polymorphic epitopes to residues 61-70 on I-Aβ chains. In a similar manner, it was shown that several diverse residues on DR β1*03 chains were involved in the integrity of an epitope common to all DR3 molecules (Mellins et al., 1990a).

Other commonly used methods for epitope mapping include DNA-mediated transfection and expression of wild-type genes, or genes that have been deliberately altered, by hemi-exon or exon shuffling or site-directed mutagenesis, into class II negative cells (Germain et al., 1985; Cohn et al., 1986; Brown et al., 1986; Braunstein and Germain, 1987; Ronchese et al., 1987). It was found that most murine I-A and I-E alloepitopes mapped predominately to the carboxyl region of the β_1 domain; however, some serological epitopes on DR molecules were found to map to other regions on DR β1 chains (Alber et al., 1989; Gorski et al., 1990; Fu et al., 1992a,b). These regions included the carboxyl-terminal and amino-terminal of the β_1 domain,

and an unidentified region on the β_2 domain.

Although the second domain of DRβ chains is highly conserved, (Bell et al. 1987; Gorski, 1989), there are sporadic polymorphic differences that have been predicted to determine some of the common serological epitopes (Gorski, 1989). For example, a DR4-specific mAb, which was mapped to the carboxyl-terminal region of DR4 β chains in a study by Alber et al., (1989) was more precisely mapped using site-directed mutagenesis to DRB1*96:E (Maurer and Gorski, 1991). This position is located at the amino end of the β_2 domain, thus strengthening the idea that the second domain controls some of the common serological specificities. It further indicated that certain predicted allele-specific residues may be critical in determining epitopes on DR molecules.

1.5.3 T-cell Defined Determinants

Most serological DR specificities include several Dw subtypes, each encoded by a distinct DRB allele. These subtypes are conventionally identified by T cell typing methods and alloreactive T-cell clones (Dupont e al, 1973; Bach et al., 1983; Groner et al., 1983; Singal et al., 1985; Flomenberg et al, 1989; Obata et al., 1992). Analysis of DRB1 sequences for Dw subtypes, clearly show that all subtypes within a generic specificity, have identical sequences with the exception of a few critical residues on the β_1 alpha helix (see Figure 1.5). Paradoxically, the same residues that differentiate

these subtypes from each another are usually homologous to that of a subtype belonging to another generic DR specificity. Again, there are few, if any allele-specific residues for T-cell defined subtypes; the uniqueness is provided by the entire sequence which may involve 20-30 residues.

This phenomenon is illustrated using some of the DR4 and DR1 subtypes (Figure 1.6). The DR4 subtypes differ from each other by one to four amino acid residues. For example, Dw14.2 (B1*0408) differs from Dw14.1 (0404) by a single conserved substitution, glycine for valine at position 86, and it differs from Dw4 (0401) by a conserved substitution, arginine for lysine at position 71. Although the differences are conservative, each of these three subtypes can be differentiated by alloreactive T cell clones (Pile et al., 1992a). In contrast Dw10 (0402) differs from the other DR4 subtypes by three to four residues. Two of these involve major non-conservative substitutions at positions 70 (negatively charged aspartic acid for an uncharged glutamine) and 71 (negatively charged glutamic acid for positively charged lysine or arginine) and the other two involve non-conservative substitutions at positions 67 (isoleucine for leucine) and 86 (valine for glycine).

The DR4 and DR1 molecules have major differences in their first two hypervariable regions. However, the HVR III sequences on the alpha helix of the different β chains reveal some interesting similarities as well as differences. For example, residues on the alpha helix that differentiate the DR1 subtypes Dw1 and Dw 'BON,' from each other are strikingly similar to the analogous sequences in

Dw14.2 and Dw10, respectively (Figure 6). In fact, Dw4, Dw14.1, Dw14.2 and Dw1 are so homologous that alloreactive T-cell clones and antibodies have been identified that react with determinants on all these molecules and any others such as Dw16, that are carry these sequences (Duquesnoy et al., 1984; Flomenberg et al., 1989; Weyand and Goronzy, 1989; Hiraiwa et al., 1990; Obato et al, 1992). This suggests that homologous sequences in these regions generate similar structures, such as the putative "shared epitope" that has been predicted to confer susceptibility to the development of Rheumatoid Arthritis (see CHAPTER 2).

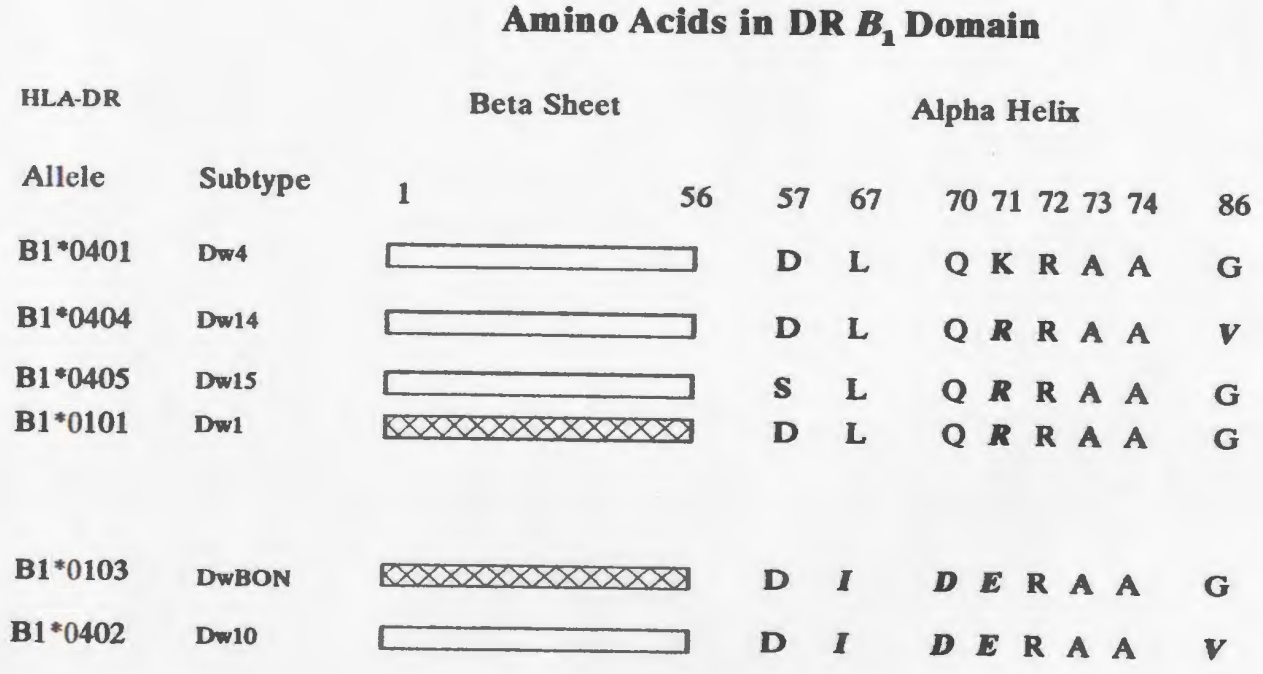

Figure 1.6 Amino acid differences and similarities between related and unrelated DR alleles. DR4 alleles (open boxes) differ from DR1 alleles (hatched boxes) at amino acids 1-56, but share sequences at positions 57-86.

1.5.4 Alloreactivity

Several studies using class II mutants and inbred mouse strains have mapped the key allodeterminants to positions 67, 70 and 71 on the alpha helix of I-A β chains (reviewed by Mengle-Gaw and McDevitt, 1985). However, due to extensive interallelic homology in this region, it seems unlikely that alloreactive T cells recognize these amino acids directly. If there are no allele-specific residues for each subtype, what then is the nature of the allo-determinant that defines a single Dw specificity? It has been suggested that the polymorphic differences on the β-pleated sheet, which forms the floor of the groove, must also contribute to the structure of the T cell determinant (Lechler and Lombardi, 1991; Obato et al., 1992). For example, two DR4 subtypes Dw13 and DwKT2 differ by only a single residue (tyrosine for serine at position 37), which lies on the floor of the groove. Since the side chains of position 37 are presumably involved in peptide binding, and since the groove contains peptide, it is likely that such (and perhaps all) alloreactive T cells recognize MHC-peptide complexes. For example the recognition of some alloreactive T cell clones has been shown to depend on the cell in which the molecule is presented, which suggests the recognition of a cell-specific peptide plus MHC (Kappler and Marrak, 1988). Further evidence for peptide being involved in the formation of an allodeterminant has come from reports of alloreactive T cells that react also with self-MHC plus a particular antigenic peptide, that is in an antigen-

specific manner (De Koster et al., 1989; Panina-Bordignon et al., 1991; Lechler et al., 1992). Thus, the general consensus is that most alloreactive T cell clones bind conformational determinants that are dependent on critical residues (notably those at positions 67, 70, 71, 74 and 86) on the alpha helix, on certain residues on the floor of the groove and on bound peptide (Lechler et al., 1992; Coppin et al., 1993; Demotz et al., 1993; Sherman and Chattopadhyay, 1993). One of the most interesting findings, which may have implications for transplantation, is that some alloreactive T cells are directed to allo-peptides that are derived from processed non-self MHC molecules and presented in the context of self-DR (Liu et al., 1992).

CHAPTER 2

HLA-DR MOLECULES AND RHEUMATOID ARTHRITIS

2.0 INTRODUCTION

An association between histocompatibility molecules and disease was first demonstrated in mice in which it was shown that certain inbred strains were susceptible to virally-induced murine leukaemia (Lilly et al., 1964). Although the first HLA-associated diseases to be identified were leukaemias and Hodgkin's disease (Amiel et al., 1967; Forbes and Morris, 1970), the majority were subsequently found to have an autoimmune component. HLA-associated diseases are generally characterized by unknown aetiology, a tendency to be inherited, but not by simple Mendelian genetics, and weak penetrance. With few exceptions, they are more closely associated with HLA-class II than with class I molecules.

The magnitude of these associations is quantitated by calculating the relative risk (RR), which is the probability of developing the disease if the particular HLA antigen is present. Thus, disease studies are done using a large sample of well-defined patients and healthy controls that are matched for gender, age and ethnic background. The formula that is used to calculate the relative risk is: $(P^+C^-)/(P^-C^+)$, where P^+ represents patients that are positive for the test antigen; C^-, controls, negative for the antigen; P-, patients, negative for the antigen, C^+,controls positive

for the antigen. Thus, a RR of 1 indicates that the test antigen does not confer any higher risk than any other antigen. A RR that is less than 1 suggests that the test antigen confers protection, while a RR that is greater than 1 suggests that the test antigen confers susceptibility to the development of the disease. A notable exception to the class II association is ankylosing spondylitis (AS) and HLA-B27, which epitomizes many of the features of HLA-associated autoimmune diseases mentioned above (Schlosstein et al., 1973; Brewerton et al., 1973). For example, although B27 is found in more than 90% of the patients with this disease, most people with B27 do not get AS. Presumably, another factor (or factors) that has not yet been defined is required to initiate and maintain the disease state. It is perplexing and frustrating that after more than twenty years since this association was made, and despite great strides in molecular immunology including the cloning of B27 genes and the production of B27 transgenic animal models, we still have very little idea of what triggers this disease or how to cure it.

From a historical perspective it is interesting to see how the study of HLA-disease associations has evolved. Such studies have been a major component of nearly all the international HLA workshops. As improvements in HLA-typing methods were made and as the complexities of HLA-genetics and linkage disequilibrium were deciphered, weak associations with class I gave way to stronger ones with class II molecules. For example, the original association of type I diabetes with HLA-A1,-B8 was later found to be more significant for DR3 and DQ2, which

are in linkage disequilibrium with A1 and B8. An even higher degree of relative risk was conferred by HLA-DR3 and DR4 heterozygosity (Svejgaard and Ryder, 1989). The molecular basis of the HLA association was further elucidated by nucleotide sequencing of certain HLA genes that were implicated in either susceptibility or protection to type I diabetes both in humans and in murine models (Acha-Orbea and McDevitt, 1986; Todd et al., 1987). Subsequently, it was shown that the absence of aspartic acid at position 57 on certain DQβ (DQ2 and DQ8) and the equivalent I-Aβ chains of I-A^{nod} mice conferred susceptibility, while the presence of aspartic acid at the same position conferred resistance to the disease.

It is beyond the scope of this review to describe the many different HLA-associated autoimmune diseases. The HLA-DR4 associated diseases, particularly Rheumatoid Arthritis are most relevant to the work that will be described in this thesis. Thus, various aspects pertaining to Rheumatoid Arthritis and the associated HLA molecules will be reviewed.

2.1 RHEUMATOID ARTHRITIS: AN OVERVIEW

Rheumatoid arthritis (RA) is a self-perpetuating autoaggressive disease of unknown etiology that afflicts approximately 1% of the population, with prevalence rates ranging from 0.1% in certain parts of Africa to to 1.6% in Finland (Spector, 1990). Like many autoimmune diseases it affects more females than males, which

suggests a role for sex hormones (Khan et al., 1988). It has a tendency to occur in families with a concordance rate of approximately 30% for monozygotic twins and 7% for dizygotic twins (reviewed by Wordsworth and Bell, 1992). However, a recent study disputes this figure and suggests that the concordance rate for monozygotic twins is only 15% (Silman et al., 1993a). It has been suggested that the concordance rates are higher when the studies are done through a rheumatology clinic than when done at the community level (Walport et al, 1992)

One of the difficulties in establishing the size of the genetic contribution is disease heterogeneity. The criteria on which current diagnosis is based were established by the American Rheumatism Association in 1987 (Arnett et al., 1988) These criteria and the various stages of disease progression have been reviewed by Harris (1990). Typically, the disease is marked by inflammation and gradual erosion of the joints with most of the immune-mediated attack focused on the synovial tissue. However, RA is not confined to the joints and it frequently affects other tissues, particularly the skin and lungs. Consequently, RA patients range from those with mild polyarthritis to those with severe symptoms, including loss of mobility and extra-articular involvement. Thus, within this generic classification of RA, there may be disease subsets in which genetics plays a further role (Deighton, 1993).

2.1.1 Immunopathogenesis of the Rheumatoid Synovium

In progressive RA the structure of the synovium closely resembles lymphoid tissue. The membrane becomes hyperplastic and is enriched in phagocytic and fibroblastic cells. Typically there is a lymphocyte-rich zone that is similar to the paracortical areas of a lymph node with CD4 positive T cells and endothelial cells predominating. Transitional areas resemble the follicular and medullary regions of a lymph node with an enrichment of B-lymphocytes and plasma cells. Uninfiltrated interstitial areas contain histiocytes, many of which are positive for class II and macrophage markers. The synovial fluid is particularly enriched in polymorphonuclear cells as well as in a variety of lymphocytes.

The predominant cells in the synovium are activated CD4 positive T cells, which express markers that are characteristic of memory cells; these include CD45RO, IL-2 receptors, class II molecules and adhesion molecules, such as LFA-1 asialyl-Lewis X and VLA-4 (Panayi, 1993; Gaston, 1993). These adhesion molecules promote binding to their respective ligands (I-CAM, ELAM-1 and VCAM-1) on endothelial cells. Furthermore, the secretion of lymphokines (IL-2 and IFN-γ) by T cells upregulates the expression of adhesion molecules, thus, exacerbating T cell infiltration into the synovium. The main effector cells are macrophages and synoviocytes which, together with the activated T cells, release a huge array of cytokines including IL-1, TNF-α, IL-6, numerous growth factors, and the

IL-2 and IFN-γ (reviewed by Panayi, 1993; Firestein and Zvaifler, 1993). In concert, these factors promote the destruction of the joints and surrounding tissue.

Approximately 70% of patients with RA are seropositive, that is, they have increased levels of anti-immunoglobulin, commonly called rheumatoid factor (RF) both in the synovium and in the circulation. RF positivity is generally associated with a poorer prognosis (Olsen et al., 1988; van Zeben et al., 1991), but there are patients who have severe RA and who are not RF positive (Calin et al., 1989). The production of RF is not restricted to RA but is found in several diseases, particularly lymphoproliferative diseases; it is also produced from activated B lymphocytes in culture. However, the variable region genes from this type of RF (usually IgM) shows restricted usage of variable region genes (Vaughan et al., 1993). In contrast the variable region genes from RF (IgM, IgG or IgA) have undergone somatic hypermutation. This suggests an ongoing antigen-driven response but, despite intensive research in this area, no antigens derived either from self-components or microbial organisms have been shown to be uniquely associated with RA.

Susceptibility to RA and expression of the disease may be coded for by several genes, particularly genes in the MHC. It has been determined that most of the genetic component is contributed by HLA, and that HLA is more important in severe progressive RA than in mild disease (reviewed in Ollier and Thompson, 1992). For example, although the vast majority of hospital-based patients with RA are HLA-DR4, this may not be the case at the community level (see section 2.5). Other non-

HLA genes that may confer susceptibility include immunoglobulin genes, notably polymorphisms that are associated with the kappa constant region (Moxley et al., 1989) and T-cell receptor genes. In the latter case, there have not been any consistent findings of particular V-region genes despite intensive research in this area (Wordsworth, 1992).

2.2 THE HLA-DR ASSOCIATION WITH RHEUMATOID ARTHRITIS

2.2.1 Historical Perspectives

The first indication of an HLA-D association with RA came from an early study by Astorga and Williams (1969), in which they clearly showed decreased or negative mixed lymphocyte reactivity (MLR) in 64% of RA when mixed lymphocyte cultures were done between patients. However, since none of the then-known HLA antigens could be correlated with altered MLR, these findings remained inexplicable. Following this discovery, HLA-D determinants were more clearly defined and it was shown that reduced MLR in RA patients correlated with an increased frequency of HLA-Dw4 in these patients (Stastny, 1976; McMichael et al., 1977). This association was confirmed by several serological studies in which HLA-DR4, which included the HLA-Dw4 specificity, was increased in the majority of RA patients (Stastny, 1978; Stastny, 1980; Christiansen et al. 1984; Zoschke et al. 1986).

The complexity of the DR4 specificity and its subtypes was first elucidated by a modified MLC using homozygous typing cells as stimulator (HTC-typing) and later by 2-dimensional gel analysis of immunoprecipitated molecules. Five different DR4 subtypes (Dw4; Dw10; Dw13, formerly DB3; Dw14, formerly, LD40; Dw15, formerly DYT) were identified by HTC-typing (Reinsmoen & Bach, 1982; Bach et al. 1983; Thompsen et al., 1983; Jarquemada et al., 1984) and immunochemical analysis of immunoprecipitated molecules (Groner et al., 1983; Nepom et al. 1983).

Analysis of the HLA-DR and Dw frequencies in RA patients of different ethnic backgrounds revealed several intriguing facts. The DR4 subtypes (Dw4 and Dw14) were most commonly associated with RA in Caucasoids living in North America and Northern Europe (Stastny, 1980); Dw4 was also found to be associated with RA in several other ethnic groups including North American blacks, Mexicans, and certain American Indian tribes (Stastny, 1980). In the Japanese population RA was associated with the DR4 subtype Dw15 (Ohta et al.,1982). In contrast the HLA-DR4 association was not found in certain populations such as Greeks and Israeli Jews; although these populations carry DR4, it is mostly the Dw10 subtype (Stastny, 1980; Amar et al., 1982; Schiff et al., 1982). In these patients from the Mediterranean region and patients of Asian Indian origin (Woodrow et al., 1981), the predominant HLA-DR antigen was found to be DR1. Furthermore, several investigators had found that DR1 was increased, secondary to DR4, in some of the Caucasian patient populations (Stastny, 1980; Christiansen et al., 1984). Thus, by the early 1980's the

HLA-association was still incomplete and obscure.

2.2.2 The Evolution of the Shared Epitope Hypothesis

Several important discoveries in the mid-eighties contributed to deciphering the HLA-association with RA. As outlined above, serology and HTC-typing had clearly shown that three DR4 subtypes (Dw4, Dw14 and Dw15) and DR1 were associated with RA in different ethnic groups, while the DR4 subtype Dw10 and possibly Dw13 seemed to be protective. Furthermore, Duquesnoy et al (1984) reported that an antigen called MC1 correlated with DR1 and most DR4 specificities, and that it was found in 93% of the patients with RA compared to 47% in normal controls. This suggested that MC1 was a better indicator of susceptibility to RA than either DR4 or DR1.

The advent of recombinant DNA technology facilitated cloning and sequencing of class II genes, which revealed regions of hypervariability that were shared among different alleles (See CHAPTER 1.). Gregersen et al., (1987) noted from examination of sequences from cloned DR1 and DR4 genes (Bell et al. 1986; Cairns et al, 1986; Gregersen et al., 1986), that the third hypervariable region of the DR1 β chain was almost identical to those of the DR4 β chains of Dw4 (0401), Dw14 (0404), and Dw15 (0405). However, the DR4 subtypes Dw10 (0402) and Dw13 (0403), which were not associated with RA, differed from the susceptible DR4

subtypes by conservative and non-conservative substitutions in this region (Figure 2.1). Moreover, the substitutions at which the "protective" Dw10 subtype differed from the "susceptible" subtypes were located at positions 67, 70, and 71. In the H-2^{b} and H-2^{bm12} murine models these positions had been previously shown to be critical in determining alloreactivity, antigen presentation, and susceptibility to experimentally-induced myasthenia gravis (Mengle-Gaw et al., 1984; Hochman, 1984).

2.2.2A HLA-DRB1* susceptibility sequence

On the basis of the discoveries and observations outlined above, a unifying hypothesis, that became known as the shared epitope hypothesis, was advanced by Gregersen et al., 1987. It predicted that the homologous sequences 67-74:LLEQKRAA/LLEQRRAA on the implicated DR β chains, generated a shared epitope that is important in conferring susceptibility to the development of RA. The same group also reported that a serologically-defined epitope (109d6) was highly associated with RA; this epitope was also present on DR10, later shown to be associated with RA in some populations (Merryman et al., 1988). Since DR10 molecules were also found to carry a similar sequence (67-74:LLERRRAA) to that of the putative shared epitope, Winchester and Gregersen (1988) suggested that the "epitope" resulting from the substitution of arginine for glutamine in the shared sequence was also positively charged and was "conformationally-equivalent" to the

epitope generated from 67-74:LLEQK/RRAA in RA. It was later shown that this 109d6 serological epitope is present on other molecules such as DR53, DR9 and DR14Dw9. Since these molecules, with the possible exception of DR9 (Massardo et al., 1990), are not associated with RA, this epitope is unlikely to be a marker of susceptibility to RA. The shared epitope hypothesis was further strengthened by the discovery that a rare DR6 allele (DRB1*1402), which encodes the same shared sequence, is highly associated with RA in two North American Indian populations (Willkins et al., 1991 and Nelson et al., 1991).

The DR subtypes that carry 67-74:LLEQKRAA/LLEQRRAA/LLERRRAA and their amino acid differences are shown in Figure 2.1. All DRβ chains have either valine (V) or glycine (G) at position 86 and several newly-identified alleles vary only by G or V at position 86 (for example, DRB1*0404 and 0408 or 0101 and 0102). Several investigators have questioned the importance of position 86 to the formation of the shared epitope and whether it is relevant in conferring RA susceptibility (Gao et al. 1991a; Nelson et al, 1991). While some studies suggested that the shared epitope plus 86G (for example, DRB1*0401,0405,0408, 0101,1001) contributed greater susceptibility, others could not confirm this (Wordsworth et al., 1992). Recently, the DR1 association with RA in Israeli Jews was shown to be greater for the variant DRB1*0102 (86V), (de Vries et al., 1993). This suggests that the contribution of position 86 to the shared epitope is minor in conferring susceptibility to RA.

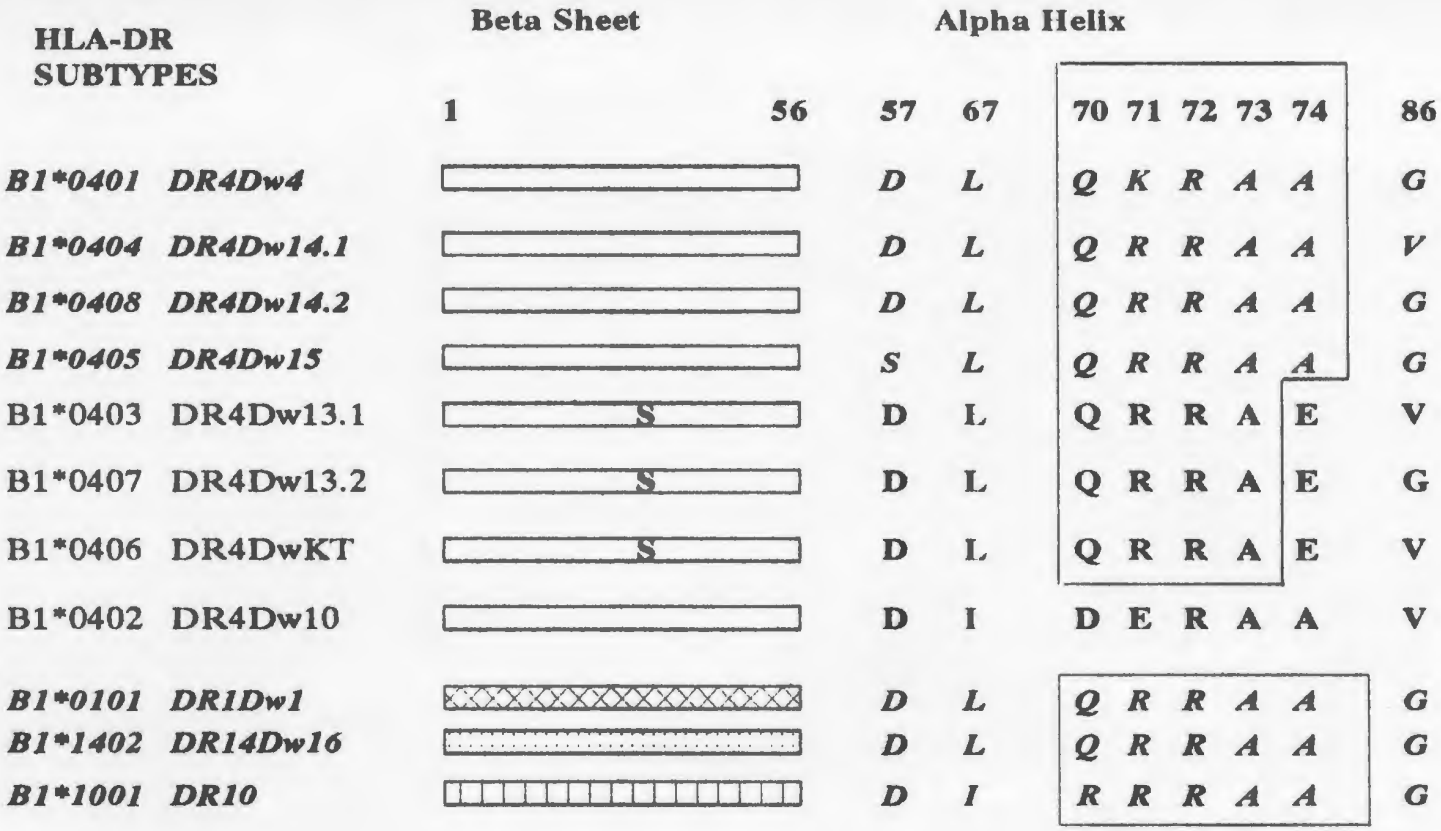

Figure 2.1 HLA-DR subtypes and association with rheumatoid arthritis (RA). The DR4 subtypes (open bars) have nearly identical amino acid sequences on the beta sheets, but differ on the alpha helix. The DR subtypes associated with RA are shown in italics and have homologous sequences (boxed) on the alpha helix; non-DR4 subtypes associated with RA differ in sequence on the beta sheets (different patterns).

2.3 HLA-DR FREQUENCIES AND THE PREVALENCE OF RHEUMATOID ARTHRITIS IN DIFFERENT POPULATIONS

DNA-based typing of class II genes using the polymerase chain reaction for gene amplification followed by the use of allele-specific oligonucleotide probes has facilitated the precise identification of the DR alleles that are associated with RA in different ethnic groups. Thus, a considerable amount of new data regarding HLA associations, allele frequencies, and the prevalence of RA in diverse populations has accumulated from disease and anthropological studies. The findings of some of these studies are summarized in Table 2.1. Wherever possible, the frequencies of the implicated DR specificities are given for the control population in the disease study and for the general population in each ethnic group.

Table 2.1 Distribution of rheumatoid arthritis, HLA-DR association and frequencies in different populations.

Ethnic Group	Associated DR Specificities and Relative Risk (RR)	Frequency of DR Specificities in the Normal Population			References
		DR4 B1*04 Alleles	DR1	DR10	
Canadian Whites	DR4;	35.1	20.2	1.2	Larsen et al., 1989
	DR4 (RR,3.4); DR1 (RR,2.9)	14.3	22.9	NA	Singal et al., 1991
		18.2 01>04/08>03	12	0.7	Imanishi et al., 1992
USA Whites	DR1; DR4 (RR,3.7); 0401(RR,6.1); 0404/8 (RR,3.7);	NA	NA	NA	Nepom et al., 1989
	DR1 (RR, 1.7) DR4 (RR,3.5); 0401(RR,5.3); 0404/8 RR, (0.9)	33.3	19.2	NA	Gao et al., 1991
		12.4 01>04/08>03	10.1	1.7	Imanishi et al., 1992

Table 2.1 continued.

Ethnic Group	Associated DR Specificities and Relative Risk (RR)	Frequency of DR Specificities in the normal population				References
		DR4	B1*04 Alleles	DR1	DR10	
UK Whites London	DR1 (RR, 1.2) DR4 (RR,10.5); 0401(RR, 11); 0404 (RR,14.3)	17	01>04>03	33	NA	Wordsworth et al,1989
Norwich	No significant increase	37		20	NA	Thompson et al, 1993
		13.9		10.7	1.7	Imanishi et al., 1992
Norwegian	DR4 (RR,7.9): 0401 & 0404	34	01>04>03	14	2	Ronningen et al, 1990
Finnish Swedish	DR4	29.5		38.6	NA	Paimela et al, 1993
	DR4: 0401 & 0404; DR1	21	01>04>03>02	10	NA	Wallin et al, 1991
Yugoslavian	DR1 (RR,2.84) DR4 (RR1.9)	34		45	NA	Jajic et al. 1992
		10.3		9.3	1.5	Imanishi et al., 1992
Italian	DR4(6.9): 0401 & 0404	10	01>04>02	15.3	4.5	Angellini et al, 1991
		7.1	01>03/07>02	8.5	1.5	Imanishi et al., 1992
Spanish	DR10 (RR,5.3); DR4 (1.8); DR1 (1.2)	26.5		20.5	4.5	Sanchez et al, 1990
	DR10 (RR,3.8); DR4 (RR,2.4): 0405 & 0401	22.7	03/07>04/08>01	23.3	3.7	Yelamos et al, 1993
		16	01>04/08>02>03	10.9	0.9	Imanishi et al., 1992

Table 2.1 continued.

Ethnic Group	Associated DR Specificities and Relative Risk (RR)	Frequency of DR Specificities in the normal population			References
		DR4 B1*04 Alleles	DR1	DR10	
Greek	DR1;DR10	14 02>01>03>04	7	5.8	Boki et al, 1992 Carthy et al, 1993
Israeli Jews	DR1 DR4: 0405 (RR,9.2)	32.8 02>03>04>05			Gao et al, 1991 Gao et al, 1991
Indian (Asian)	DR4	26.1	14.1	2.2	Taneja et al, 1993
Indian (UK)	DR10 (RR 3.8); DR1 (3.4)	11.9	6	8.9	Ollier et al, 1991
		10.6 04>03>02/01	5.6	5.6	Imanishi et al., 1992
S. Chinese	DR4 (RR, 3.4): 0404>0405	17.8 05>04	0	4.4	Seglias et al, 1992
	DR4 (RR,4.6):	14 05>06>04>02	0	5	Molkentin et al. 1993
Han Chinese	DR4 (RR,2.5); DR1 (RR,6.8)	15	1	1.5	Imanishi et al., 1992
Thai Chinese	DR4 (RR,2.2)	16.4	0	2.1	Imanishi et al., 1992
Japanese	DR4:0405 (RR,3.3)	22.8 05>06>03/07	5.5	0.6	Tsuchiya et al.,1992 Imanishi et al., 1992

Table 2.1 continued.

Ethnic Group	Associated DR Specificities and Relative Risk (RR)	Frequency of DR Specificities in the normal population			References
		DR4 B1*04 Alleles	DR1	DR10	
N.Zealand Polynesians	DR4: 0405 & 0404 (RR,11)	26 03>01>04/05	NA	NA	Tan et al, 1993
S.Africa Negroids Cape Town		2.8 01>04>05	2.8	1.7	Imanishi et al., 1992
	DR4 (RR,3.9)	13	NA	NA	Martell et al, 1989
		10.4	4.7	2.6	Imanishi et al., 1992
Zimbawean	DR4	8	14	6	Martell et al, 1990
		1.9	9.2	3.8	Imanishi et al., 1992
Sotha	DR4 (RR,12.9)	9.3	NA	NA	Pile et al, 1991
Zula	DR4 (RR,59)	7.1	NA	NA	Pile et al, 1991
Kuwait	DR3 (RR,23.56)	NA	NA	NA	Sattar et al, 1990
Chile	DR9 (RR,9.3)	29	16	NA	Gonzales et al, 1992
Yakima Indians	DRB1*1402 (RR,3.3)	NA	NA	NA	Willkins et al, 1991
Tlingit Indians	DRB1*1402 (RR,2.4)	21	2	2	Nelson et al, 1992

2.3.1 Correlation of RA Prevalence with Increased HLA-DRB1*0401 Frequency

An intriguing observation (Winchester, 1992) which is clear from the studies summarized in Table 2.1, is a North to South trend for both the frequency of DRB1*0401 and the prevalence of RA. For example, Caucasoids living in North America and Northern Europe have a high DRB1*0401 frequency and an increased prevalence of RA, while Caucasoids living in certain Mediterranean countries have a low DRB1*0401 frequency and a reduced RA prevalence. In Spaniards, Greeks and Israeli Jews, RA is more frequently associated with DR10 and/or DR1. Recent studies have also implicated DRB1*0405, which was unexpected since the frequency of DRB1*0405 in those populations is lower than for other DR4 alleles. As previously noted the frequency of "protective" alleles, Dw10 (0402) and Dw13 (0403/0407), is significantly higher in these countries than in Northern Europe.

The controversy regarding the HLA-association with RA in India has not been completely clarified from these studies. For example, Ollier et al., (1991) and others have found that RA in Indians living in the UK is associated primarily with DR10 and DR1. However, Taneja et al (1993) and the 11th IHW studies (summarized by Nelson et al, 1991) found a positive association with DR4, specifically DRB1*0404. These differences are likely to reflect the size and racial diversity of the population of India. For example, in the 11th IHW anthropology study the frequencies of DR4 were 4.3% for Punjabis in New Delhi; 18.6%, for the Iyers in South India; and

11.7% for Bhargavas in North India; mixed Indians living in Canada, the United Kingdom and USA had a frequency of 10.8 percent (Singal et al, 1992).

The prevalence of RA and the frequency of DR4 and DRB1*0401 in Africa are quite variable (Modyetal, 1989; McGill, 1993), but again they more or less parallel each other. For example, both DR4 and RA are virtually non-existent in Nigeria (Silman et al., 1993b); in South African Negroids the prevalence rate ranges from 0.2% to 0.9%, with the highest RA prevalence and DRB1*0401 frequency being found together in urban regions such as Soweto. It would be of interest to know the RA prevalence rate in one of the Negroid tribes (San Bushman), which has a DRB1*0401 frequency of 44.8%. It has been pointed out, that the prevalence rates given for RA in Africa may be diluted by a larger population of younger people than that which is seen in Europe (McGill, 1993; Pile et al., 1992b).

Japan has a moderate prevalence of RA (0.6%) despite having a high frequency of the "susceptible" DRB1*0405; it is noteworthy that the DRB1*0401 frequency is less than 1 percent. Similar or reduced prevalence and an increased DRB1*0405 frequency have also been reported for southern China and the Thai Chinese (Silman et al; 1992). Furthermore, in New Zealand Polynesians in which the high frequency of DR4 is due to the allele B1*0403, the prevalence of RA is reported to be low and it is associated with DRB1*0404 and 0405 (Tan et al., 1993).

2.3.1A Frequency of the shared epitope in ethnic groups

Since susceptibility to RA is closely associated with the putative shared epitope (DRB1*67-74:LLEQKRAA/LLEQRRAA/LLERRRAA), several of the studies summarized in Table 2.1 reported the frequency of the "susceptibility sequence" in RA patients and controls. As might be predicted the highest frequency of this sequence was found in Northern Caucasoid RA patients (80% to 93% compared to about 40% in the control populations). Frequencies in Southern Caucasoids patients were generally lower, with the lowest being reported in a study of Greek patients in which 43.5% of RA patients had the sequence compared to 15.5% of the controls (Boki et al., 1992). However in another study involving Greek patients, the sequence was present in 60.5% of the patients, compared to 22.1 % of the controls (Carthy et al.,1993). This finding is a little lower than that found in Italians (64.5% for patients versus 29.3% for controls) and Spaniards (68.6% versus 37%), (Angellini et al. 1991; Yelamos et al. 1993). In Jewish and Indian patients, the frequency of the susceptibility sequence accounted for about 56% of the patients (Gao et al. 1991; Ollier et al., 1991). The frequency of the susceptibility sequence in one study of South African Blacks (78% for patients compared to 24% for controls) closely parallels that found in northern Caucasoids (Pile et al., 1991), while the frequency in Orientals is significantly lower (roughly 40% versus 15%), Seglias et al., 1992; Molkentin et al., 1993). As has been previously pointed out, the Yakima Indians and Tlinglit Indians have an exceptionally high frequency of DRB1*1402

(61% in control populations) and have a high prevalence of RA (greater than 85% of the patients having DRB1*1402). Cerna et al., (1993) have recently shown that in native South American Indians in which HLA polymorphism is restricted, the frequency of DR14 *1402 and 1406 is also high.

2.4 ASSOCIATION OF HLA CLASS II MOLECULES THAT DO NOT CARRY THE SUSCEPTIBILITY SEQUENCE WITH RHEUMATOID ARTHRITIS

Aside from DR molecules carrying a version of the susceptibility sequence, very few DR molecules have been consistently associated with RA susceptibility. However, a significant association with DR3 in an Arabic population was recently reported (Sattar et al, 1990). It has also been shown that DR3 is associated with gold-induced cytotoxicity in RA patients (Panayi et al., 1978; McCusker et al., 1991). DRβ1*0301 shares glutamine and lysine at positions 70 and 71 with DRβ1*0401, but differs from the susceptibility sequence at positions 73, 74, and 77. It is generally considered to be neutral in conferring RA susceptibility, but based on these reports it may be associated with some subsets of RA. It is interesting that Larsen et al. (1989) found that DR3 in combination with DR4 was significantly increased in females with RA, whereas DR3 in combination with DR7 was "protective".

HLA-DR53 is also associated with RA, but it has been suggested that this is

due to linkage disequilibrium with DR4. The evidence for it not being associated with RA is based on the fact that DR53 is also in linkage disequilibrium with DR7 and DR9. DR7 appears to be negatively associated with RA (Ronningen, 1990; Nelson et al., 1992), but DR9 has recently been shown to be significantly increased in RA patients in a Chilean population (Gonzales et al., 1992). This raises the issue of whether DR9 and DR53 have "conformationally equivalent epitopes" as was proposed by Winchester and Gregerson (1988). This idea was partly formulated on the presence of a serological epitope for the monoclonal antibody (109.d6) on DR53 and DR10 molecules, which together with DR9 and DR14 (β1*1401) molecules carry part of the "shared susceptibility sequence". It is also of interest that an Italian study also found an increase in DR14 in RA patients, although this increase was not significant. The main difference between the susceptibility sequence on β chains for DR10 and those for DR9, Dw9, and DR53 is a glutamic acid substitution for alanine at position 74, which has been predicted in the DR4 subtypes (Dw13 and DwKT2) to confer protection. Winchester et al. (1992) have recently suggested that, if the association of DR9 (which also has a phenylaline substitution at position 67) with RA is confirmed, then positions 67 and 74 might not be that critical for susceptibility, thus limiting the consensus sequence to 70-73:(QKRA/QRRA/RRRA). Perhaps then, the earlier suggestion by Winchester & Gregerson (1988), that DR53 in association with a susceptible DR4 (or DR9) molecule increases RA susceptibility, has some merit.

The association of RA with DQ3 (DQ7 and DQ8), which is in linkage disequilibrium with DR4, is still somewhat controversial. It has been suggested that DQ7 molecules are also implicated in the development of RA (Singal et al., 1987; McCusker & Singal 1988; Lanchbury et al., 1989). In contrast, others found that DQ7 and DQ8 are equally distributed in RA patients (Wallin et al., 1988; Wordworth et al., 1989; Nepom et al., 1989; Gao et al., 1990, Angellini et al., 1991). In fact, Taneja et al. (1993) found that DQ8 was significantly associated with RA patients of Asian Indian origin, while DQ7 was not. Although some studies support the idea that DQ7 is implicated in severe RA (Stephens et al., 1989; Singal et al., 1992b), it is possible that this finding merely reflects the fact that DRB1*0401, which is in linkage disequilibrium with DQ7 and DQ8, is associated with disease severity. For example, virtually all patients with Felty's syndrome, which is a subset of severe RA carry DRB1*0401 and usually DQ7 (Lanchbury et al.,1991; Wallin et al., 1991).

2.4.1 Susceptible and Protective Haplotypes

Much of this review has focused on the association of class II molecules with susceptibility to the development of RA. However, as previously mentioned the DR4 molecules, Dw10 and Dw13 molecules are associated with decreased risk for RA. In addition other class II molecules have been shown to be negatively associated with RA, but this "protectiveness" is not consistent for all the ethnic groups. For example,

DR2 (DR15 and DR16) has been found to confer protection in Northern Caucasoids (Young et al., 1984; Jaraquemada et al., 1986; Stastny et al., 1988; Ronningen et al., 1991), while the same phenomenon was not found in studies of Greek patients (Carthy et al., 1992). Two recent studies on HLA associations with mild and extra-articular forms of RA revealed that DR2 is significantly more common in mild forms than in severe forms (Singal et al., 1992b; McMahon et al. 1993).

Other HLA-DR molecules that are associated with protection include DR11 (11th. IHW); DR7 (Stastny et al., 1988; Ronningen et al., 1990); DRB1*0803 (11th IHW); DR6 (Stastny et al., 1988; Ronningen et al., 1990, 11th IHW). Certain phenotypes containing DR7 (DR3,DR7; and DR5,DR7) were also found to be significantly associated with protection (Larsen et al., 1989). In the latter report, the authors predicted the existence of a hybrid DQ molecule (DQA1*0501 from the DR3 or DR5 haplotype combined with DQB1*0201 from the DR7 haplotype), might confer protection by affecting T cell selection. This is an interesting hypothesis since the DQB1*0201 allele from DR7 haplotypes differs from that of the DR3 haplotype by a substitution at position 135 in the second domain (Hall et al., 1992); they have predicted that a similar heterodimer might confer susceptibility to the development of Coeliac disease .

Since DQ specificities are in linkage disequilibrium with several different DR alleles, it is instructive to review the DQ alleles on "susceptible" haplotypes and "protective" haplotypes. These are summarized in Table 2.2 and it is apparent that

some DQ specificities, including DQ7 and DQ8, are found in both groups. For example, the DQA*0501 and DQB1*0301 alleles, which encode DQ7 on the DRB1*1402 haplotype are positively associated with RA (Nelson et al., 1992), while the same alleles on the DR11 haplotypes are associated with protection. This refutes the notion that the DQ7 molecules encoded by DQA1*0501 might be protective (McCusker and Singal, 1988). Furthermore, the DQ7 specificity that is encoded by A1*0301 and B1*0301 is associated with susceptibility on the DRB1*0401 haplotype, but associated with protection on DRB1*0407 haplotype. Similarly, DQ8 is associated with susceptibility (DRB1*0401 and 0404) and with protection (DRB1*0402). It has been proposed that DQ2 might be protective (Ronningen et al., 1990) but its presence on certain DRB1*0405 haplotypes (Awad et al, 1990) which are associated with RA, would argue for DR7 being the protective molecule.

The only consistent finding in Table 2.2 regarding DQ alleles is that those encoding DQ6 specificities are almost always associated with protection. However, the DR alleles that are found on these haplotypes have major differences in the putative susceptibility sequence. Thus, DQ molecules do not appear to confer susceptibility or protection, but several DRB1 alleles that encode aspartic acid at position 70 are frequently associated with RA.

Table 2.2 HLA Class II Haplotypes associated with A) Susceptibility or B) Resistance to the development of Rheumatoid Arthritis.

A) Susceptibility

DRB1*	DRB1*67-74	86	DQA1*	DQB1*	DQ
0401	LLEQKRAA	G	***0301***	***0301***	7
0401	LLEQKRAA	G	***0301***	***0302***	8
0404	LLEQRRAA	V	***0301***	***0302***	8
0405	LLEQRRAA	G	***0301***	0401	4
0405	LLEQRRAA	G	***0301***	***0201***	2
1402	LLEQRRAA	G	***0501***	***0301***	7
0101	LLEQRRAA	G	0101	0501	5
0102	LLEQRRAA	V	0101	0501	5
1001	LLERRRAA	G	0101	0501	5

B) Resistance

0402	ILEDERAA	V	***0301***	***0302***	8
0403	LLEQRRAE	V	***0301***	***0302***	8
0407	LLEQRRAE	G	***0301***	***0301***	7
0701	ILEDRRGQ	V	0201	***0201***	2
0803	ILEDRRAL	G	0103	0601	6
1101	FLEDRRAA	G	***0501***	***0301***	6
13	ILEDERAA	V	0102 0103	0603 0604 0605	6
1501 B5*01	ILEQARAA FLEDRRAA	V G	0102	0601	6

Boldface lettering indicate amino acid residues that differ from those in DRB1*0401; boldface and italics, indicate DQ alleles that are associated with both susceptibility and resistence.

2.5 SUSCEPTIBILITY VERSUS SEVERITY

The vast majority of studies that have shown a significant association of DR4 and/or DR1 with RA have been carried out on hospital-based patients. Some of the very early studies suggested that DR4, in particular Dw4, was associated with disease severity. For example, it was found that 75-80% of hospital based RA patients had DR4 (Stastny, 1978; Roitt et al, 1978; Panayi et al., 1978), while DR4 was only marginally associated with RA in those patients who were grouped together on the basis of ARA criteria (Stastny, 1980 IHW). In a prospective study of RA in the UK, it was found that DR4 was significantly increased only in those patients who progressed to erosive bone disease as detected by radiographic changes (Young et al., 1984). A community-based study in the Netherlands, showed no significant increase of DR4 (de Jongh et al., 1984).

The idea that the presence of DR4 in these patients might be a marker for severity was strengthened by two different studies, which showed that more than 90% of hospital based patients who developed severe extra-articular forms of the disease had DR4 compared to 78% (Ollier et al., 1984) and 63% (Westedt et al., 1986) of those who did not. Several studies showed that patients who were seropositive, which is generally an indicator of disease severity, were usually DR4 (Stastny, 1980, Stastny et al., 1988; Olsen et al., 1988). However, in a comprehensive study, by Calin et al., (1989) in which they compared two sets of patients who were matched for age,

sex and bone destruction, but differed in seropositivity, they showed that DR4 was equally prevalent (69%) in both groups. They suggested that DR4 might be a marker for disease progression and severity.

As serological methods and cumbersome cellular methods were replaced by DNA typing, a more accurate analysis of the HLA genes that are involved in susceptibility or severity has been made. Much of this has already been reviewed with an overall consensus that both DRB1*0401 and 0404 are most often associated with severe forms of RA (Nepom et al., 1986; Hillarby et al., 1991; Lanchbury et al., 1991). It appears that Dw14 has a synergistic affect on Dw4; recently Nepom (1992) reported that the risk ratio for developing clinical RA in the white population is 1/7 for DRB1*0401/0404, 1/35 for 0401 or 0404; 1/46 for 0401/00101 or 0404/0101 compared to 1/580 for the general population. It is intriguing, however, that in Greek and Oriental populations DR4 is not associated with severity and that the DR4 subtypes associated with RA in these populations are Dw14 and/or Dw15, but not Dw4.

Recent reports have firmly supported the idea that predisposition to disease severity is greatest when the patient carries two copies of the susceptibility sequence, particularly in the DR4 context. For example Wordsworth et al, (1992) have calculated a hierarchy of risks in the following order: 0401/0404 > 0401/0101 > 0404/0404 > 0401/0401 >0404/0101 >04/x ~ 0101/0101 >01/x > x, where x is any allele except 0401/0404/0101. Weyand et al (1992a, 1992b) also found that

homozygosity for the susceptibility sequence in the DR4 context was associated with extra-articular features, joint replacement and the worst prognosis. DR4 homozygosity was also shown to be significantly associated with severe radiological changes in RA patients who did not have extra-articular features (McMahon et al.41, 1993).

2.5.1 DR4 as a Prognosis Marker?

Retrospective studies have strongly suggested that DR4 may be useful in predicting patients who will progress to severe RA. Thus DR4 and particularly DR4 genotyping may be a useful prognosis marker, but it needs corroborating by doing prospective studies. A few prospective studies have already been done, but unfortunately in the majority of these studies the patients were not DR-genotyped. The results have been somewhat controversial and inconclusive.

An early study (Young et al., 1984) found an association between DR4 and the development of bony erosions, while another (de Jong et al., 1984) found no association. Of four prospective studies that have been recently reported in which DR subtyping was not done, one suggested that DR4 is a useful predictor for disease progression (van Zeben et al., 1993), two showed a significant increase in DR4 in the RA population, but one of these suggested that it was not useful for prognosis (Paimela, 1993); the other suggested that it was limited to predicting large joint

damage (Eberhardt et al., 1993) In a large community based study in Norwich, DR4 was not significantly increased over that found in the normal population, and it was suggested that DR4 is only associated with long term RA (Thompson et al., 1993). A small fifth study was recently reported in which DR genotyping was performed (Salmon et al., 1993). They showed that about half of the patients who presented with RA-like polyarthritis developed classical or persistent RA and these patients had a significant increased frequency of Dw4 compared to the patients who did not develop classical or severe RA. However, Dw14 or DR1 were not increased in either group, which suggest that Dw4 may be associated with persistent forms of RA.

The difficulty with all of these studies is that due to the heterogeneity of RA it is hard to establish whether DR4 or any other marker is useful for prognosis when it is likely that there is no HLA association with mild forms of polyarthritis that happen to fit the ARA criteria for RA. Thus, it has been suggested that there may be two main subsets of the disease, HLA-linked and non-HLA-linked (Deighton et al., 1993a, 1993b). The HLA-linked would include poor prognosis, extra articular involvement, RF positivity, a significant association with the susceptibility sequence and inheritance.

CHAPTER 3

THESIS PROPOSAL AND RESEARCH PLAN

When this thesis project was first proposed, the direction of much of the efforts in the field of HLA immunogenetics and HLA-associated diseases, was based on T-cell and molecular methodology (10th International Histocompatibility Workshop). However, it was felt that a major contribution in this area could be made by making monoclonal antibodies to the DR4 subtypes; these subtypes were known to be important in transplantation and in conferring susceptibility to certain autoimmune diseases, notably Type I Diabetes and Rheumatoid Arthritis.

The belief that monoclonal antibodies could be made, at least against some of the subtypes, was partly based on the identification of a cluster of alloantisera in the 9th International Histocompatibility Workshop which produced three different DR4 serological patterns. In addition biochemical variants were described that correlated with the T-cell defined subtypes. Most importantly, a shared determinant (MC1), which was found on the DR molecules that are associated with RA, was identified both by alloantisera and T cell clones. Coincidently, transfectants expressing class II genes had been described and these appeared to be a considerable improvement over the usual B cell lines as immunogens.

It was anticipated that anti-DR4 mAbs would be useful for tissue typing, thus

contributing to the field of transplantation. In particular, it was envisioned that they would be valuable in the study of the HLA-association with Rheumatoid Arthritis, as tools for testing the "shared epitope" hypothesis advanced by Gregerson et al., (1987). It had been proposed that non-DR4 individuals who develop RA have epitopes shared with DR4 on their DR. If this were proven to be correct, then antibodies that recognize such epitopes would be useful for identifying individuals at risk for RA and for probing the molecular structure of these disease-associated determinants.

3.0 SPECIFIC AIMS AND OBJECTIVES

(i) to produce monoclonal antibodies that recognize epitopes on DR4 molecules and that differentiate the various DR4 subtypes

(ii) to produce monoclonal antibodies that recognize shared structures on the DR molecules that are implicated in susceptibility to the development of rheumatoid arthritis.

(iii) to use these monoclonal antibodies to search for "shared epitopes" in rheumatoid arthritis patients

iv) to use these monoclonal antibodies to map the critical residues that are involved in the formation of the "shared epitope."

3.1 STRATEGY FOR THE DEVELOPMENT OF THE MONOCLONAL ANTIBODIES

To optimize the conditions for making the anti-DR4 mAbs, it was decided to forgo using human B cells for immunization in favour of mouse cell transfectants, which express a single type of HLA molecule. It was envisioned that mice immunized with these cells would make a significant response to the foreign HLA molecule with an amplification in the number of antigen-specific B cells available for fusion.

It was decided to make these DR4-expressing transfectants using DNA-mediated transfer of genomic DNA into L cells which had proven to be successful for the expression of HLA class I and CD molecules. This became unnecessary when a set of transfectants were generously donated by Dr. R Sekaly and Dr. R. Karr.

Previously described fusion methodology (MSc thesis, Drover, 1986) was modified so that approximately one hybridoma per well in a 96-well microtitre tray was obtained, thus simplifing the interpretation of the screening results.

A previously developed screening method, cellular enzyme-linked immunoassay (CELISA) was adapted for fast differential screening of hybridoma supernatants using transfectants that adhered to the plastic wells of a microtiter tray.

CHAPTER 4

TRANSFECTION OF HLA GENES USING GENOMIC DNA[1]

4.0 ABSTRACT

Mouse L cells expressing HLA genes are potentially useful for producing and analyzing monoclonal antibodies (mAb) to HLA molecules. This paper describes the preparation of transfectants using uncloned human DNA and three methods to isolate the HLA-expressing transfectants. Transfectant libraries were made by co-transfecting mouse thymidine kinase (tk) deficient L cells with a calcium phosphate precipitate containing genomic DNA and tk plasmid DNA. Transfectants expressing HLA-genes were isolated using these methods: immunomagnetism, replicate-plating combined with CELISA, and sorting using a FACS. Two HLA-A2 transfectants were isolated using immunomagnetism, two HLA-A24 transfectants by replicate plating and one HLA-Bw60 transfectant by FACS. However, no transfectants were isolated that stably expressed class II genes. The class I transfectants have been useful in characterizing several locally prepared mAbs which bind to monomorphic determinants on class I HLA molecules. Two of the transfectant lines, one expressing HLA-A2 (8001) and the other HLA-A24 (8008), have been included in the collection of lines distributed for use in the 11th International Histocompatibility Workshop.

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[1]Published: Sheila Drover and William H Marshall, Human Immunology 31: 293,1991.

4.1 INTRODUCTION

Production of monoclonal antibodies (mAbs) in the mouse to HLA polymorphisms has proven to be difficult largely because mice have been immunized with impure mixtures of antigens. The use of mouse cells expressing cloned HLA genes should improve the technology, since only one foreign molecule is presented to the mouse (Heyes et al., 1986). Using this technology to produce monoclonal antibodies to all the HLA polymorphisms would require cloning and transfection of more than 150 HLA genes. This appears to be an impractically large undertaking.

The use of whole uncloned human DNA to make stable transfectants has been described by various authors; for example, Kavathas and Herzenberg, (1983) showed that about 1/1000 of such transfectants express HLA class I. This prompted us to attempt to transfect class II HLA genes in the same manner.

4.2 MATERIALS AND METHODS

4.2.1 Cell Lines

Human B-cell lines were maintained in RPMI-1640, with 10% fetal bovine serum (FBS), and antibiotics. Two EBV-transformed cell lines, derived from patients with multiple sclerosis were used to prepare genomic DNA. FS-6467 was HLA-typed

as A2,3; Cw4,w6; B14,44; DR2,4,w53; DQw1,w3 and FS-6845 typed as A3,24; Cw6,w7; B7,w60; DR2,4,w53; DQw1,w3.

Two sublines of the tk negative C3H mouse fibroblast L cell line were used. The H subline was given to us by H. Hamada; the K subline, was obtained from P. Kavathas. Both were maintained in Dulbecco's Modification of Eagle's Medium (DMEM)* containing 10% FBS, 2-mercaptoethanol and antibiotics.

4.2.2 Co-Transfection

Genomic DNA was isolated from the B cell lines, FS-6467 and FS-6845, as described (Kavathas and Herzenberg, 1986). The plasmid pHSV106 (BRL Life Technologies, Inc., Burlington, Ont. Can. L7P1A1) carrying the tk gene was co-transfected with the genomic DNA into tk negative C3H L cells (Kavathas and Herzenberg, 1986). Briefly, the DNA (20 μg genomic DNA to 200ng pHSV106) was calcium-phosphate precipitated and immediately added to a monolayer of L-cells, in a 100 mm tissue culture dish (Becton & Dickinson, Lincoln Park, NJ, USA 07035). Four hr later the cells were shocked for 3 min with 15% glycerol, washed with DMEM and returned to growth medium. In some experiments the cells, at 20 hr after glycerol shock, were trypsinized and plated at 5000 cells per well in 96-well flat-bottom Linbro plates (Flow Laboratories, Inc.). Transfectants were selected by feeding with HAT* medium (Kavathas and Herzenberg, 1986) at 48 h and every

three days from then on.

4.2.3 Antibodies

The mAbs we used are shown in Table 4.1. They were either ascites fluid or supernatants from overgrown hybridoma cultures and were optimally diluted in medium. The secondary antibody used in CELISA was affinity purified F(ab')2 fragments of goat anti-mouse immunglobulin conjugated to horse radish peroxidase (GAM-HRP), (Jackson Immunoresearch Laboratories Inc West Grove, PA 19390). For cell sorting and flow cytometry the secondary antibody was either FITC-labelled whole molecule IgG goat anti-mouse IgG + IgM (FITC-GAM), (Coulter Immunology Hialeah, FL. USA 33010), or dichlorotriazinyl amino-fluorescein-labelled affinipure F(ab')2 fragments of goat antimouse IgG + IgM (DTAF-FITC), (Jackson Immunoresearch Laboratories, Inc.).

4.2.4 Immunometallic Bead Technique (Immunomagnetism)

Transfectants were harvested at three weeks by trypsinizing the cells for 10 min. The cells were then left in 10 ml HAT medium in non-tissue culture petri dishes (to which they do not adhere) for 24 hr before assay.

Table 4.1. Monoclonal antibodies used in the isolation and selection of HLA-expressing transfectants.

mAb	Specificity	Source/reference
NFLD.M1	anti-DR4 plus	Local/Drover et al. (1985)
NFLD.M2	anti-A2 + A28	Local/10th IHW 2021
NFLD.M6	anti-DR	Local/ Marshall et al. (1986)
NFLD.M15	anti-class I	Local/unpublished
NFLD.M16	anti-class II	Local/unpublished
NFLD.M44	anti-A24	Local/unpublished
W6/32	anti-class I	ATCC/Barnstable et al. (1978)
BB7.6	anti-Bw4/w6	ATCC/Brodsky et al. (1982)
L243	anti-DR	ATCC/Lampson and Levy (1980)
IVD12	anti-DQ3	ATCC/Giles et al. (1983)
GAP A3	anti-A3	ATCC/van Schravendijk et al. (1983)
Tu 39	anti-class II	10th IHW, Knowles et al., 1989
MB40.3	anti-B40 + B7	ATCC/Parham (1981)
B27.M1	anti-B27 + B7	ATCC/Grumet et al. (1981)

ATCC, American Type Culture Collection

For isolating and selecting HLA-expressing transfectants, 2 x 10^6 transfectants in 2% FBS/DMEM were reacted with 0.5 ml of a cocktail consisting of three mAbs recognizing class-II determinants: NFLD.M1, NFLD.M6, & NFLD.M16, as well as two against HLA Class I: NFLD.M15, and NFLD.M2 (see Table 1). The mixtures were rotated at room temperature for 60 min, washed 3 times and resuspended in FBS/DMEM. Immunometallic beads (Lea et al., 1985) coated with goat anti-mouse IgG (Dynal Inc., Great Neck, NY 11021) were added at a ratio of 3 beads per cell and rotated in the cold for 30 min. Harvesting was done by washing with 10 ml FBS/DMEM and trapping rosetted cells on the side of the tube using a cobalt magnet (Dynal Inc.). After four washes the trapped cells were resuspended in 1 ml HAT medium, counted and subcultured at 2000 cells/well in a 24-well plate (Linbro). This was followed by cloning by limiting dilution. An alternative method used in a few experiments was to directly rosette the colonies using mAbs and immunometallic beads. Single colonies were removed with trypsin inside a cloning ring.

4.2.5 Replicate Plating and CELISA

Transfectants seeded in a 96-well flat-bottom microtiter plate were allowed to grow to confluency and then, after trypsinization, three replicate plates were made. The cellular enzyme-linked immunoassay (Drover and Marshall, 1986) was used to identify wells containing HLA-expressing transfectants, by using a mAb cocktail

(W6/32, BB7.6, L243, IVD12 and NFLD.M6) or an individual mAb (See Table 4.1). Positive cultures were cloned by limiting dilution from the third replicate plate and positive wells again identified by a CELISA on replicate plates.

4.2.6 Cell Sorting and Flow Cytometry

In some of the later experiments transfectant libraries were sorted using a Coulter EPICS-CS FACS. Five million cells were reacted aseptically with 0.5 ml of a mAb cocktail consisting of Tu39 and W6/32 for 45 min on ice. This was followed by three washes with 2% FBS in DMEM and staining with 500 μl of GAM-FITC (Coulter). After the final wash dead cells were stained with propidium iodide (Sigma), analyzed and sorted essentially as described by Kavathas & Herzenberg (1983). The cells were sorted at 5000 cell/sec and collected at 100, 50 and 10 cells per well in 96-well Linbro plates using the Autoclone attachment. Transfectant clones isolated by immunomagnetism, replicate plating and cell sorting were also analyzed by flow cytometry, using various mAbs and and staining with DTAF-GAM.

4.3 RESULTS

4.3.1 Isolation and Analysis of HLA-Expressing Transfectants

4.3.1A HLA.A2

HLA-expressing cells were sorted from three transfections using immunomagnetism. After transfection, the number of tk positive colonies surviving the HAT selection was approximately 500 per million cells plated. Between 1% and 3% of cells were rosetted in the three experiments (1.6%, 2.6%, 1.0%) and the magnetic sorting procedure resulted in an apparent enrichment of up to forty fold (68%, 53%, 15%). In control experiments less than 0.5% of non-transfected L cells bound three or more beads. The isolated cells (a mixture of rosettes and non-rosettes) and beads were plated at 2000 cells/well in 24 well plates. The cells isolated from one experiment (BL2) grew well but for unknown reasons cells from the other two failed to grow. Some days later they were recultured in limiting dilutions in 96 well plates, having either 100, 50, 25 or 12.5 cells per well. There was growth in all wells. Replicate plates were made and CELISA tests done, which showed positive results (O.D. above 0.5 after background subtraction) in all wells seeded with 100/well, in 92% of wells seeded at 50/well, in 68% of wells seeded at 25 cells/well, and in 25% of wells seeded at 12.5 cells/well. This data suggests that approximately one cell in eight that had been plated out after immunomagnetic

TABLE 4.2. Comparison of the expression of HLA on the transfectants to HLA expressed on homozygous B cell lines using CELISA and a panel of monoclonal antibodies.

		mAb						
Cell	HLA-ACB	W6/32 1/10	BB7.6 1/5	NFLD. M2 1/10	NFLD. M44 1/10	GAP.A3 1/10	MB40.1 1/1	B27.M1 1/1
BL2-8G-E6	A2	0.42[a]	0.02	1.02	0.02	0.01	0.01	0.01
K2B-12H-G7	A24	0.57	-	0.01	1.90	0.02	0.01	0.01
WG50-F3	Bw60	0.98	1.31	0.04	0.01	0.02	1.33	0.01
JESTHOM	A2;Cw10;B27	2.4	2.4	1.82	0.01	0.04	0.35	0.78
DKB	A24;Cw10; B40	2.5	1.96	0.03	2.13	0.09	1.73	0.23
SAVC	A3;Cw7;B7	2.4	1.89	0.05	0.04	0.80	1.92	0.18

[a] OD values after the background was subtracted.

enrichment was a stable HLA expressor.

Further cloning and testing resulted in a clone, BL2-8G-E6, whose reactions with a panel of antibodies (Table 4.2 and Figure 4.1) indicated that it was expressing HLA-A2, the key reaction being with NFLD.M2 an antibody characterized in the 10th Workshop (Table 4.1).

In addition to the HLA-A2 transfectant described above, a second one was isolated from a different experiment using the K subline of L cells. This yielded 1400 colonies which were directly rosetted on the plate using the same mAb cocktail and immunometallic beads. Two rosetted colonies were removed using a cloning ring. One survived the cloning procedures and was shown to express A2 (data not shown).

4.3.1B HLA.A24

The replicate plating method together with the CELISA assay was used to isolate an HLA-A24 transfectant called K2B-12H. Cells from a transfection done using genomic DNA from FS-6845 and the K subline of L cells, were seeded at 5000 cells per well in 96 well microtiter dishes. Colonies grew in all 288 wells. Replicate plates showed that two wells gave high O.D. values, (1.33 and 2.0) with the antibody cocktail. Only the latter survived the subsequent cloning procedure and was shown to express HLA-A24, the key positive reaction being with NFLD.M44 which is considered specific for HLA-A24 (by extensive testing on cell lines), together with negative results with GAP A3 (anti-A3) and the B locus mAb BB7.6 (Table 4.2).

4.3.1C HLA.B7/40

A library of transfectants made from genomic DNA derived from FS-6845 was reacted with a mAb cocktail (W6/32 + Tu39), stained with FITC-GAM and sorted using the FACS. The sorted cells were plated at 10, 50 and 100 cells per well. Between 33%-63% of the wells grew cells and those were replica plated. Eight wells gave strong positive results with W6/32; those cells were also positive with BB7.6 (B locus) but negative with antibodies to A3 and A24 (data not shown). Since the DNA donor was HLA-B7 and B40 (subtype 60), the cells were tested to see which of these genes had been successfully transfected. Using the mAbs MB40.3 and B27.M1, which are specific for HLA B7 + B40 and for HLA B7 + B27, respectively, it appeared that these cells were expressing the gene for HLA-Bw60 (Table 4.2).

4.3.2 Analysis of HLA Expression on the Cloned Class I Transfectants

4.3.2A Flow cytometry

Each of the cloned transfectants was tested with the anti-class I mAb (W6/32), and a mAb recognizing the appropriate HLA specificity. Culture medium served as a negative control. It is apparent from Figure 4.1 that the majority of cells in both the BL2-8G E6 and K2B-12H G7 populations are high expressors and most likely clonal. The WG50-4C F3 population is more heterogenous with a large proportion of the cells being high expressors and a small proportion of the cells being negative.

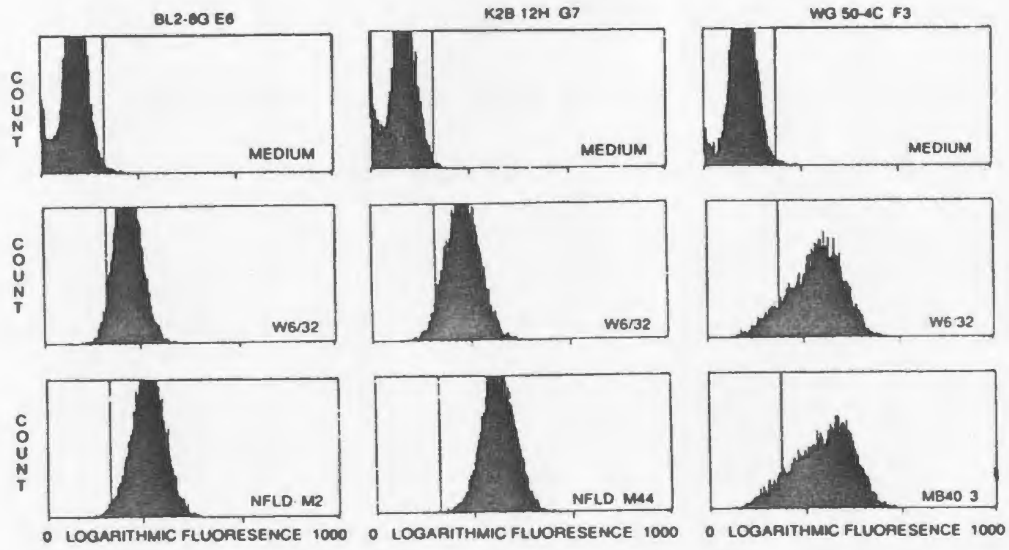

Figure 4.1 Shows flow cytometry analysis of the expression of class I molecules on the transfectants. Each of the cloned transfectants was reacted with medium (negative control) shown in the top panel, and with the anti-class I mAb (W6/32), shown in the second panel. Each of the transfectants was also reacted with a mAb specific for the molecule expressed (shown in the third panel).

4.3.2B CELISA

A panel of mAbs was used to compare the expression of HLA on the transfectants to that on B-cell lines. It can be seen from Table 4.2 that in each case the specific antibody gave a lower optical density in CELISA with transfectants than it did with the appropriate B cell line; calculating the difference in terms of percentage of OD, shows 56% for A2, 89% for A24 and 73% for Bw60. This is a crude comparision and does not directly correlate with the number of molecules expressed per cell. In the case of W6/32, which binds to all class I molecules, the difference at this dilution is considerably larger; however it should be remembered that while B cell lines express HLA-A, HLA-B and HLA-C molecules, the transfectants are presumed to express only one of these.

4.3.3 Use of HLA-Class I Transfectants for Characterizing Monoclonal Antibodies to Class I Monomorphic Determinants

The three class I transfectants have proved useful in characterizing some locally-prepared mAbs; these react with all human B cell lines tested except Daudi, a class I negative line, suggesting that they are antibodies against monomorphic determinants on class I molecules (data not shown). CELISA was done in which four of our mAbs plus W6/32 and BB7.6 were tested on the three transfectants (Figure 4.2). As expected W6/32 (anti-HLA-ACB) reacted with all three transfectants

whereas BB7.6 (anti-B locus) was positive only with WG50 (HLA-Bw60). NFLD.M23, NFLD.M25, and NFLD.M26 produced OD values comparable to that produced with W6/32, suggesting that these are directed to an epitope on the heavy chain. NFLD.M15 reacted only with the Bw60 transfectant and not with the A2 or A24 transfectants, and reacted in a manner similar to the BB7.6 control. This suggests that it is directed to an epitope on the HLA-B heavy chain.

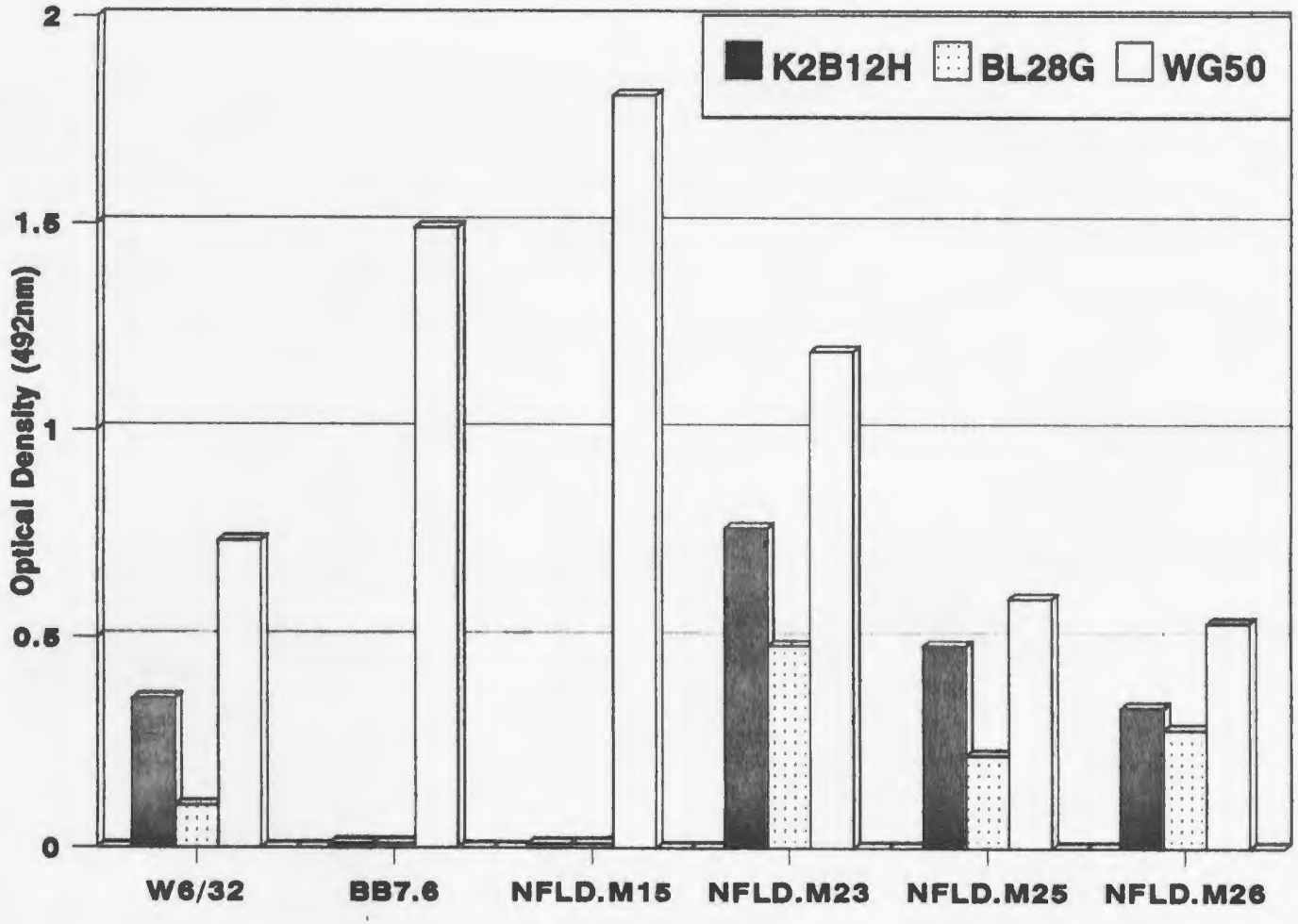

Figure 4.2 The specificities of anti-class I mAbs (NFLD.M15, NFLD.M23, NFLD.M25, NFLD.M26) were analyzed by CELISA using K2B-12H G7 (HLA-A24),BL2-8G E6 (HLA-A2),and WG50-4C F3 (HLA-Bw60). W6/32 and BB7.6 were used as controls.

4.4 DISCUSSION

It is well documented that L cells are good recipients for the expression of cloned class I and class II genes (Lemonnier et al., 1982; Germain and Malissen, 1986). Although we were successful in making class I transfectants, our initial goal in these experiments, to produce mouse cells that expressed HLA class II molecules, was not reached. There was no stable expression of class II, although there was some evidence of transient expression of class II genes in a few experiments (data not shown). Possibly a recipient cell that expresses mouse class II molecules might be a more appropriate recipient, for example a mouse lymphoma or leukaemia line. The transfection of class I genes by this method may turn out to be the more important aspect since there are by now considerably more cloned class II genes available in expression vectors than there are for class I.

An estimate of the frequency of transfection of class I genes can be made from our data. Among the cells surviving HAT selection that were tested and isolated using immunomagnetism, approximately 2% appeared to bind anti-HLA antibodies in the initial set of experiments. When these cells were sorted from the rest and cloned, about one in eight developed into a stable transfectant. By calculating backwards and ignoring the possibility of artifacts in the sorting and rosetting methods, we can estimate that about one in 400 of the original cells became stably transfected with the HLA-A2 heavy chain gene. When immunometallic beads were

used to directly rosette the colonies, 2/1400 colonies were rosetted, giving a frequency of 1/700. The same frequency was obtained using the replicate plating technique, in which it was found that two out of 288 wells contained class I expressors, in an experiment in which there were probably 1400 transfectants (about five tk transfectant colonies per well); thus about 1/700 transfectants was expressing a class I gene. This estimate is in line with that calculated by Kavathas & Herzenberg (1986).

Our experiments never showed evidence for simultaneous transfection of an HLA-A and an HLA-B gene; we had no reagents to see if HLA-C and HLA-B had been transfected together, which would be not unlikely, given the closeness of those two genes. The transfection of two unlinked genes e.g. two different HLA-A genes or an HLA gene and a beta 2-microglobulin gene would be a rather rare event and we found nothing to suggest this might have happened; the latter was excluded by testing with BBM1 (Brodsky et al., 1979), an anti beta 2 microglobulin antibody (data not shown).

This method, which relies on simple yet effective technology brings the production of transfectant cells within the reach of any tissue typing laboratory that has cell culture capability. Expensive instruments such as a FACS are not required.

Two of the transfectants described here are in the collection circulated for use in the 11th International International Workshop; they are BL2-8G-E5 (8001) and K2B-12H (8008).

CHAPTER 5

USE OF TRANSFECTANTS AS IMMUNOGENS FOR MAKING ANTI-DR4 MONOCLONAL ANTIBODIES [1]

5.0 ABSTRACT

Monoclonal antibodies (mAbs) that are specific for DR serotypes and subtypes have been difficult to make using human B cell lines as immunogens. The work presented here describes efforts to make mAbs using mouse cell transfectants, which express a single DR4 specificity, as immunogens. Several different immunization schedules were tried including neonatal tolerance, which failed. The first eleven fusions produced 6500 hybrids, of which one was DR4-specific. Fifteen fusions done to find the the optimal immunization scheme, produced over 8000 hybrids, of which thirteen made antibodies to DR4 polymorphisms. The largest number of specific mAbs were derived from mice that had been given a single injection of cells, either subcutaneously in CFA or intraperitoneally, followed by an intrasplenic boost three days prior to fusion. Eight antibodies that were selected have the following preliminary specificities: NFLD.D1, DR4-specific; NFLD.D7, pan-DR4, DR2 and DR52; NFLD.D2, NFLD.D3, NFLD.D4, NFLD.D8, NFLD.D9 and NFLD.D10 all recognize subsets of DR4, excluding Dw10, as well as certain non-DR4 molecules that are usually associated with Rheumatoid Arthritis.

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[1] Shorter version published: Sheila Drover, Dianne Codner, Jane Gamberg, and William H Marshall. In: Tsuji K, Aizawa M, Sasazuki T (eds): HLA 1991, Vol 1. Oxford. Oxford University Press, 1992.

5.1 INTRODUCTION

Efforts to make murine monoclonal antibodies (mAbs) that differentiate the HLA antigens have proved it to a be difficult task. This is partly due to the immunogen, B cells or B cell lines which express an enormous array of different molecules including at least six different HLA antigens. The murine immune system recognizes most of these molecules as foreign and even when purified HLA molecules are used, the majority of the antigen-specific cells recognize the species-specific or monomorphic determinants present on histocompatibility molecules (Bodmer et al., 1984).

The availability of mouse L cell transfectants expressing HLA genes as immunogens is a promising alternative since, conceptually, the only foreign molecule expressed on the surface of the transfectant should be an HLA molecule. Therefore, it is expected that some of the antigen-specific B cells in a C3H mouse immunized with such transfectants will be directed to HLA molecules and that some of these should be against polymorphic determinants. Since only one kind of HLA molecule is expressed on an L cell transfectant, the task of differentially screening hundreds of hybridoma supernatants for specific antibody in a short time, is enormously simplified.

The use of a transfectant by Heyes et al., (1986) to produce anti-DP mAbs, including one against a polymorphic epitope, was encouraging for those interested

in producing mAbs with the ability to differentiate between allelic variants previously only defined by T cells. For example, the DR4 specificity consists of several variants including eight T-cell defined subtypes (Bodmer et al., 1990). The work presented in this paper describes efforts to produce mAbs to HLA-DR4 molecules using transfectants expressing various DRB1*04 alleles. The complete characterization and epitope mapping of these mAbs is described elsewhere **(see CHAPTERS 6 AND 7).**

5.2 MATERIALS AND METHODS

5.2.1 Transfectants

The DR4 transfectants used for the immunizations and most of the analysis (Table 5.1) were generously provided by Robert Karr and Raffi Sekaly and were later included in the transfectants distributed by the organizers of the 11th International Histocompatibility Workshop (IHW) (Inoka et al., 1991). They were all made using mouse L cell of C3H origin. They include L89.2. (Dw13); L164.11 (Dw10); L165.6 (Dw14), all of which were generously donated by Robert Karr. The Dw4 transfectant (DAP3-DR4) which was used for most of the immunizations was a kind gift from Raffick Sekaly along with three non-DR4 lines.

Table 5.1 HLA-DR expressing transfectants obtained from the 11th International Histocompatibility Workshop and used for limited specificity analysis.

11th IHW No.	HLA Specificity	Local Name	Contributor
8103	DR1	DAP3 DR1	R Sekaly/E Long
8107	DR2aDw2	DAP-3DR2a	D Jaraquemada/E Long
8109	DR2bDw2	DAP-3DR2b	D Jaraquemada/E Long
8111	DR2bDw12	LARB1	H Inoko
8112	DR3	L168.2	R Karr
8115	DR4Dw4	DAP-3DR4	R Sekaly/E Long
8116	DR4Dw10	L164.11	R Karr
8118	DR4Dw14	L165.6	R Karr
8123	DR4DwTAS	L89.2	R Karr
8125	DRw11Dw5	L91.7	R Karr
8126	DRw14Dw9	L167.2	R Karr
8127	DR14Dw16	L182.1	R Karr
8134	DRw52bDw25	DAP-3DR52b	R Sekaly/E Long
8205	DQ2αDQβ	L21.	R.Karr
8305	DP4.2	L25.3	R.Karr

All transfectants were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin and streptomycin (all from Flow Laboratories, McLean, VA 22102) 5 x 10^{-5} mM 2-mercaptoethanol (Sigma, St. Louis, MO 63178). The cells were grown in either 10 cm dishes (Becton Dickinson, Lincoln Park, NJ 07035) or 75cm flasks (Linbro, Flow Laboratories), and were harvested in log phase using trypsin (Flow Laboratories,) and then left in ordinary bacteriological petri dishes (to which they do not stick) for one to three days before use. Expression was assayed with the monoclonal antibody, Tu39, which is specific for monomorphic determinants on class II molecules (Knowles et al., 1989), using either a cellular enzyme-linked immunoassay (CELISA) or indirect immunofluorescence and flow cytometry as previously described (see **CHAPTERS 4 and 6.**).

5.2.2 Immunizations

Mice were either procured from Charles River Canada Inc., St. Constant, Quebec or bred in the local animal facilities. Cells expressing high levels of DR molecules (optical density (OD) of at least 2.0 in CELISA with one of the above anti-DR mAbs) were washed three times with phosphate-buffered saline (PBS) and in all immunization procedures 1 x 10^7 cells were injected. Essentially three strategies were tried (Tables 5.2 to 5.4). The first consisted of indiscriminate standard-type immunizations where young adult C3H mice were immunized two or three times

intraperitoneally (IP) over a period of 1 to 6 months followed by a final boost intravenously (IV) or IP three days prior to fusion (Table 5.2). In the second approach neonatal tolerization was attempted. Groups of mice (3 to 5 to a group) were injected with 1 x 10^7 non-DR4 transfectants, (a mixture of DR2, DR5 and DR52) at less than 24 hours after birth and at various times, thereafter (Table 5.3). Finally, the third group consisted of 10 wk old female mice which were primed either IP in saline or subcutaneously in complete Freund's adjuvant (CFA), left for 4 to 6 weeks and then boosted either IP or intrasplenically (IS) three days prior to fusion (Table 5.4). For all these experiments the transfectant DAP-3.DR4, which expresses the Dw4 subtype was chosen because 1) an antibody to Dw4 was most desirable, 2) its DR4Dw4 expression was excellent and 3) it tested mycoplasma free. In several experiments sera were collected before and /or at the time of fusion and were tested for specific antibody in CELISA.

5.2.3 Fusion

All fusions were done three days after the final boost and were carried out using the fusion partner SP2/0-Ag14 (Shulman et al., 1978). Hybridization was done according to a previously described method (Drover, 1986) and the fused cells were plated in 96-well flat-bottom microtiter trays (Linbro). In two experiments cells were seeded at various concentrations and with different feeder cells, in order to establish

optimal conditions. Selection was done using standard HAT reagents in DMEM containing 20% FBS and supplements as previously described (Drover, 1986).

5.2.4 Screening & Specificity Testing

All supernatants were tested approximately 10 days after fusion using CELISA as previously described (Drover, 1986; Drover and Marshall, 1986). For the first screen the supernatants were tested against the immunogens; all positives were then differentially screened on the following day against the immunizing cell and non-transfected L cells. Those that were positive only with the immunizing cells were selected for further testing against a small panel of transfected cells including those expressing DP, DQ and informative DR. Hybridomas were then selected for freezing, cloning and further analysis on both transfectants and B cell lines as described as previously described (Drover, 1986).

5.3 RESULTS

5.3.1 Optimal Culture Conditions

The first fusions were carried out using the culture conditions that had been found to be satisfactory for fusions derived from Balb/C mice, namely plating at a density of (3-5 x 10^5 cells/well) together with feeder cells consisting of 2 x 10^7 red blood cells (RBC)/ml and 2.5 x 10^6 splenocytes/ml (Drover, 1986). However, the hybrids that grew in the initial fusions were rapidly overgrown and appeared to be killed by cells resembling small lymphocytes.

Two experiments were devised to check the plating density and the use of different feeder cells, in order to overcome this problem. The feeder cells were all from C3H mice and included splenocytes (S), red blood cells (R), peritoneal exudate cells (P) and thymocytes (T). The numbers of surviving hybrids in all wells were counted on days 10, 12, 15 and 17.

The density of the fused cells per well greatly affected the number of surviving hybrids (Figure 5.1) Plating at a density of greater than 2 x 10^5/well created conditions which led to the loss of the majority of the hybrids as early as day 10 with virtually none alive by day 15. Although a plating density of 2 x 10^5 per well yielded a high number of hybrids (approximately 400/10^8 splenocytes), the number significantly decreased by day 15.

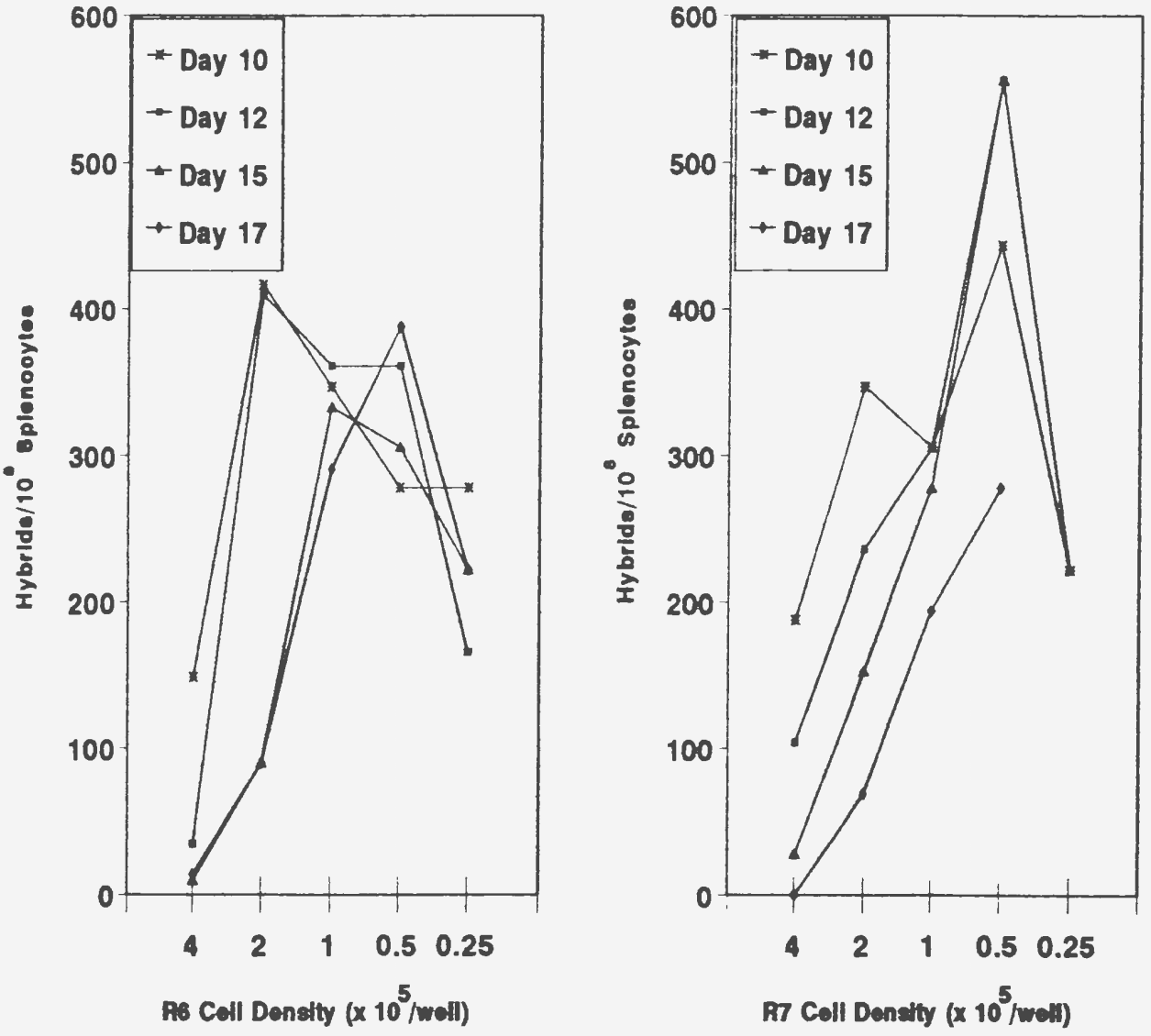

Figure 5.1 Effect of cell density on the number of hybrids per 10^8 spleen cells. Two different fusions (R6 and R7) were plated at five different densities as shown on the X axis. Hybrids were evaluated by phase contrast microscopy on days 10, 12, 15 and 17.

At a plating density of less than 2 x 10^5, the actual number of hybrids per 10^8 splenocytes increased and they survived for a longer period. However, at 5 x 10^4 or even 1 x 10^5/well, the number of wells that would have to be plated per spleen would be unmanageable; for example, an immunized spleen can yield as many as 4 x 10^8 cells, which would generate 4000 to 8000 wells at such a low plating density.

The choice of feeder cells was important (Figure 5.2). Thymocytes were consistently poor feeders in FB-plates, but in U-bottom plates, thymocytes were supportive when the fused cells were plated at low density (< 0.5 x 10^5 cells/well) but inhibitory when plated at high density (>1 x 10^5) (data not shown). Other feeder cells were not significantly different from each other in their ability to promote or inhibit growth, although PECS seemed slightly better while RBC's were less effective when the cells were seeded at a low density.

After reviewing the data it was decided to plate the fused cells at 1.5 x 10^5 to 2 x 10^5 cells per well in flat-bottom plates, to use RBC's as filler cells and to test the supernatants by day 10. Careful monitoring of the hybrids using phase-contrast microscopy was done after 1 week and hybrids were usually tested on day nine or day ten. It is evident from the data presented in Tables 5.2 to 5.4 that these conditions were sufficient to generate about 350 hybrids/10^8 splenocytes seeded for each fusion.

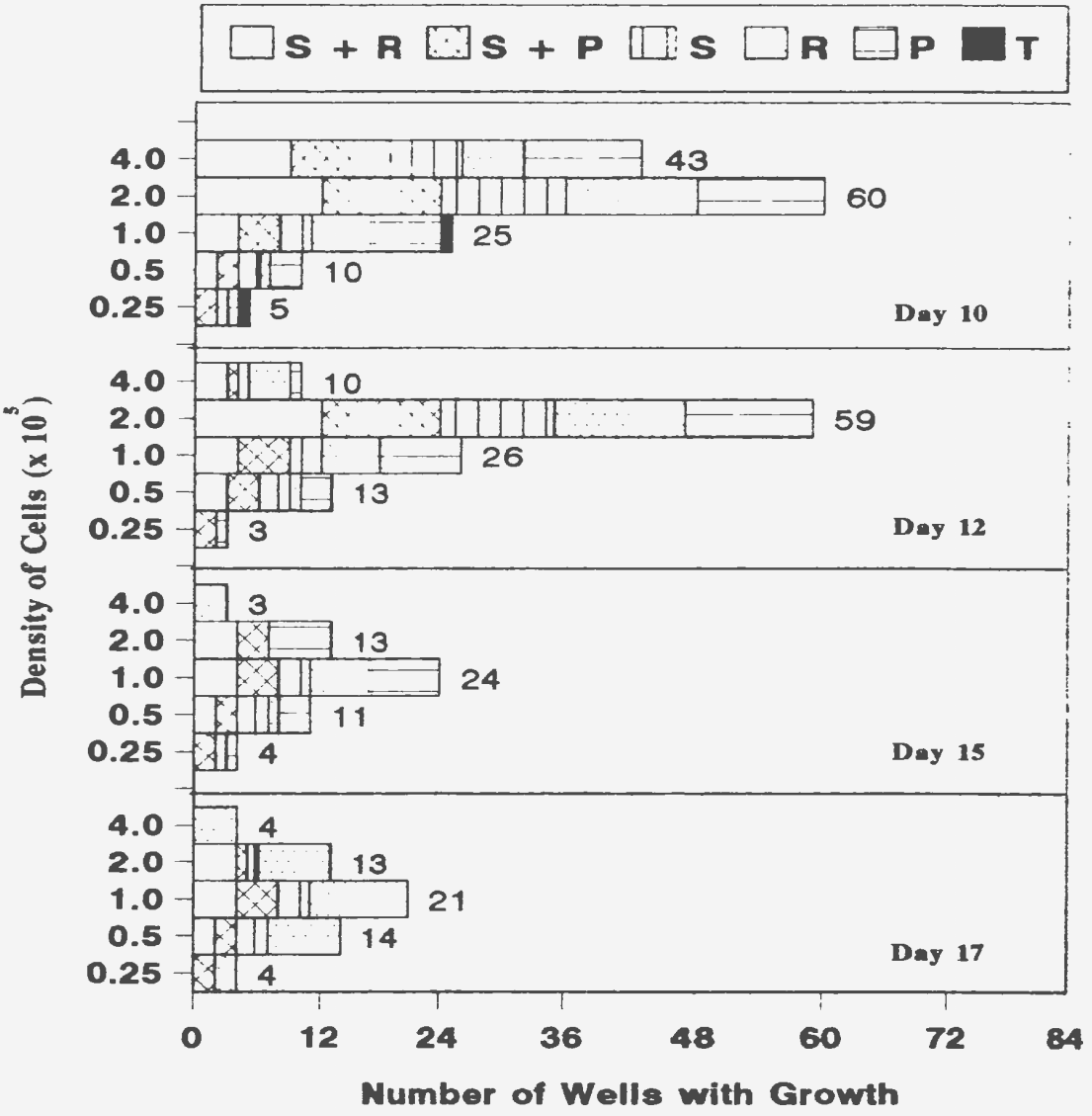

Figure 5.2 Effect of using different feeder cells (S, splenocytes; R, red blood cells; P, peritoneal exudate cells; T, thymocytes) in promoting the growth and survival of fused cells plated at five different densities. The number of wells with viable hybrids were estimated by phase contrast microscopy on days 10, 12, 15 and 17.

5.3.2 Indiscriminate Immunizations

In the first set of experiments, summarized in Table 5.2, five fusions were derived from mice that had been randomly immunized. Supernatant fluid from over 2500 hybrid cultures were assayed on the immunizing cells. After differentially screening the positive wells, seven were further analyzed for polymorphic specificity. As can be seen from the data, there were no hybrids that made antibodies with a short specificity; two antibodies had polymorphic activity but were "broadly reactive" and were of low affinity. Five hybrids produced antibody to class II monomorphic determinants. None of these were considered worth following and were discarded.

5.3.3 "Neonatally tolerized" Mice

An attempt was made to tolerize neonatal C3H mice by injecting them with non-DR4 transfectants, which consisted of a mixture of transfectants expressing DR2 and DR52. Cells were given IP within 24 hours of birth and at various times thereafter, as shown in Table 5.3. Serum samples were collected from a group of 5 "tolerized" mice and 3 non-tolerized littermates at about 7 weeks and titered in CELISA against the tolerizing cells and non-transfected L cells.

Table 5.2 Summary of data on fusions derived from C3H mice given indiscriminate immunizations with HLA-class II expressing transfectants.

Fusion	Sex	Immunization Schedule: Age (wks)	Immunizations	Boost	# Hybrids[a] (per 10^8 splenocytes)[b]	HLA-specific[c] hybrids
R5	M	10 12 14	1° DR1 IP 2° DR1 IP 3° DR1 IP	DR1-IP 5 wks after last boost	75 (214)	1 DR monomorph
R12	M	2 16 32	1° DR4 + CFA S/C 2° Dw4 IP 3° Dw4 IP	Dw4-IP 4 wks after last boost	860 (478)	1 DR monomorph 2 long polymorphs
R13	F	20 28	1° Dw10 IP 2° Dw10 IP	Dw10-IV 3 wks after last boost	1015 (423)	2 DR monomorphs
R14	F	20 28	1° Dw14 IP 2° Dw14 IP	Dw14-IV 3 wks after last boost	204 (82)	1 DR monomorph
R15	F	20 28	1° Dw14 IP 2° Dw14 IP	Dw14-IP 3 wks after last fusion	336 (280)	NONE

[a] total number of wells with hybrids; [b] approximate number of hybrids per 10^8 spleen cells; [c] based on limited specificity analysis

Table 5.3 Results of fusions done using "neonatally tolerized" C3H mice and non-tolerized littermates

Fusion	Sex	Immunization Details: Age at injection	Cells used to tolerize	Cells used to immunize	Boost details	# hybrids[a] (per 10^8 splenocytes)[b]	Selected Hybrids[c]
R6	M	<24hrs	DR52 + DR2				
		2 wks					1 weak
		4 wks			Dw4IP	412	polymorph
		6 wks			@ 17 wks	(258)	1 DR
		8 wks		Dw4 + CFA			monomorph
		14 wks		Dw4 IP			
R7	M	<24hrs	DR52 + DR2				
		2 wks					
		4 wks					
		6 wks					
		8 wks		Dw14 + CFA	Dw4 IS	273	2 weak
		14 wks	Dw14 IP	@ 17 wks	(273)		polymorphs
			NONE				
R8	F	8 wks		1° Dw4 + CFA	Dw4 IP	750	1 monomorph
		16 wks		2° Dw4 IP	@ 20 wks	(469)	1 weak
							polymorph
				NONE			
R9	M	8 wks		1° Dw4 + CFA	Dw4 IP	750	1 monomorph
		16 wks		2° Dw4 IV	@ 20 wks	(444)	1 weak
							polymorph
R10	M	24 hrs	Dw10		Dw13 - ?	1250	
		16 wks		Dw13 + CFA	@ 28 wks	(288)	NONE
		20 wks		Dw13 IP			
			NONE				
R11	M	16 wks		Dw13 + CFA	Dw13	600	1 anti DR4
		20 wks		Dw13 IP	@ 28 wks	(462)	3 monomorphs

[a] total number of wells with hybrids [b] approximate number of hybrids per 10^8 spleen cells; [c] selected on basis of limited specificity testing

The CELISA data showed evidence of antibody activity in the sera of the "tolerant" mice, but not in the sera from control littermates, to the "tolerizing" cells and to non-transfected L cells, indicating that tolerance had not been achieved (data not shown). The sera were then adsorbed twice with L cells and once with the "tolerizing" cells and tested on DAP-3 DR52 and DAP-3 DR4Dw4 transfectants (Figure 5.3). It is apparent that there was no difference in the antibody titer against DR52 (one of the "tolerizing" cells and DR4Dw4, not used for tolerizing), which strongly suggested that the mice were not tolerized to the DR molecules.

It was decided to use some of these mice for fusions. Thus at age 8 to 16 weeks, they and some of their non-tolerized litter mates were immunized with DR4Dw4 or Dw13-expressing transfectants. The data in Table 5.3 show that very few hybrids were selected for further studies despite screening over 4000 hybrids. Three fusions from the "tolerized" group produced 1935 hybrids, of which one reacted with a monomorphic determinant and 3 reacted weakly with polymorphic determinants.

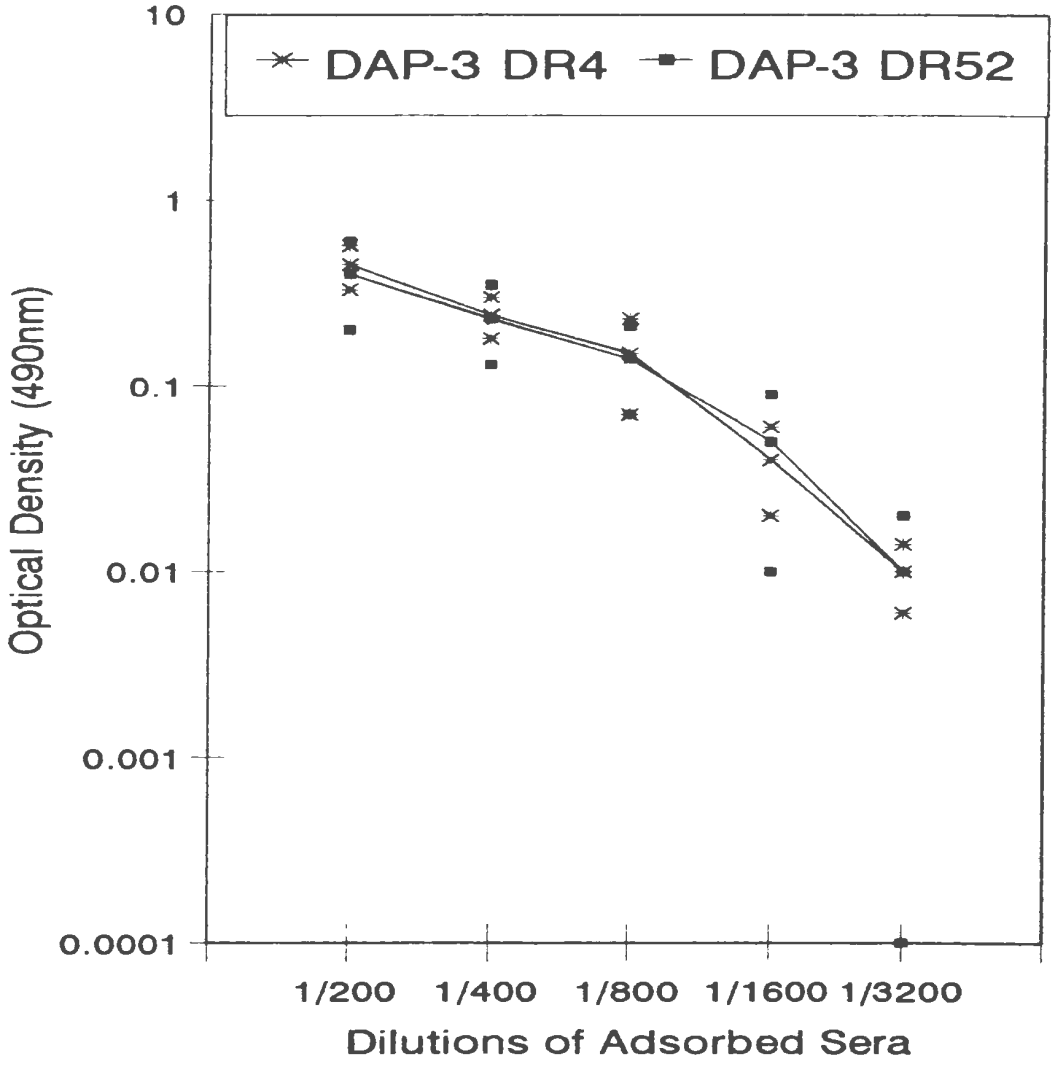

Figure 5.3 Adsorbed sera from mice that had received non-DR4-expressing transfectants, as described under methods, were titered in CELISA using DAP-3 DR4 and DAP-3 DR52 transfectants as targets. Lines connecting symbols represent the mean of CELISA data from five different sera; unconnected symbols represent one standard deviation above and below the mean.

From the non-tolerized group three fusions yielded 2100 hybrids of which 5 had monomorphic specificity, 2 were weakly reactive with polymorphic determinants and 1 reacted with all DR4 molecules. The reaction pattern and epitope mapping for this last antibody (derived from the R11 fusion and now called NFLD.D1) is described in Reference 10 and CHAPTER 6.

5.3.4 Comparative Immunizations

Fifteen fusions that were done to compare different immunization schedules were very productive. The data summarized in Table 5.4 clearly show that the worst immunization strategy was an IP primary followed by an IP boost. The best result was obtained by immunizing subcutaneously along with CFA followed by an IP boost, while the other two strategies immunizing IP in saline or subcutaneously with CFA followed by an IS boost were equally good. Titering of sera, taken at the time of splenectomy, was not always predictive of the number of specific hybrids. For example the mouse (fusion R21) that produced the highest titer against the immunizing cells produced a large number of hybrids, but did not yield a single antigen-specific hybridoma, while the mouse (fusion R16) which had the lowest titer produced 7 antigen-specific hybridomas. On the otherhand, the most prolific fusion (R19) was derived from a mouse with one of the highest titers; thus, titering of sera at this stage was not a reliable indicator of the number of specific hybrids.

Table 5.4 Results of fusions done to compare immunization schedules.

Fusion	Immunization: Primary	Immunization: Time Interval (weeks)	Immunization: Boost	# Hybrids[a] (per 10^8 splenocytes)[b]	Number of Hybrids Selected	Post-fusion Serum Titer[c]
R16	IP	4	IS	1128 (434)	7	<1/100
R22		5		336 (480)	2	1/1600
R27		6		238 (170)	5	1/400
R29		7		467 (275)	3	1/800
				X = 542 (340)	X = 4.3	
R18	IP	4	IP	864 (455)	0	1/800
R20		5		41 41	1	1/800
R26		6		546 (455)	1	1/800
				X = 483 (317)	X = 0.7	
R17		4		758 (237)	1	>1/1600
R23	CFA	5	IS	38 (23)	3	>1/1600
R25	S/C	6		409 (340)	4	1/1600
R30		7		592 (423)	4	1/3200
				X = 449 (258)	X = 3	
R19		4		812 (427)	18	1/3200
R21	CFA	5	IS	672 (354)	0	>1/3200
R24	S/C	6		560 (467)	9	>1/800
R28		7		704 (440)	7	>1/3200
				X = 687 (422)	X = 11	

[a] total number of wells with hybrids; [b] approximate number of hybrids per 10^8 spleen cells
[c] (OD of test serum with Immunogen) - (OD of test serum with L cells) = 0.5 was arbitrarily chosen as the end point of titer.
IP = Intraperitoneal; CFA = Complete Freunds Adjuvent ;
S/C = Subcutaneous; IS = Intrasplenic; X = Average

From fifteen different fusions, yielding over 8000 hybrids, sixty-four were selected for further analysis (Table 5.4). Thirteen produced antibody to polymorphic determinants. Eight of these were selected for complete specificity analysis using transfectants and homozygous cell lines. There were several patterns of reactions including: (i) Dw4, Dw14, DR1 and DR14 (Dw16); (ii) Dw4, Dw14, Dw13 (weak), DR1, DR14 (Dw16) and DR2; (iii) DR4 (except Dw10), DR1, DR2, and DR14; (iv) all DR4 molecules and DR2, plus DR52. A complete description and analysis of these antibodies is in press (see **CHAPTER 6**).

5.4 DISCUSSION

It was found that the conditions that had been used for previous fusions, which were done using Balb/c splenocytes and Balb/C-derived fusion partner, SP2/O-Ag14 (Drover, 1986) were not suitable for fusions using spleen cells from C3H mice. Since these mice are H-2 incompatible, it is possible that allogeneic T cells from the C3H spleen (unfused cells from immunized spleen and feeder cells) were cytotoxic for the Balb/c SP2/O-Ag14 fusion partner, which express class I antigens. The failure of feeder T cells to support hybrid growth supports the idea that T cells were responsible for hybrid cell death during the early phase of the culture period particularly when wells were seeded at a high cell density. Ideally, feeder cells should be X-irradiated, but by plating at a density of less than 2×10^5 fused cells/well and

using red cells as feeder cells, the problem of hybrid death was almost completely eliminated. In most of the subsequent fusions 80-90 % of the wells produced hybrids with a yield of about 350 hybrids/10^8 splenocytes. In later experiments (data not shown), T cell depletion of the spleen cells prior to fusion combined with using irradiated feeder cells allowed for plating the cells at 1 x 10^5 cells/ well and increased the number of hybrids per fusion by almost two-fold.

Although a large number of hybrids were obtained from the the set of fusions shown in Table 5.2, none were obtained that made DR4-specific antibody. The idea to tolerize mice to the monomorphic determinants on the HLA molecules expressed on syngeneic transfectants seemed reasonable, since the immune system of a neonatal mouse is immature and thus should be susceptible to tolerance induction. It was also anticipated that the syngeneic L cells might survive for a long period, thus increasing the likelihood of inducing tolerance. However, this was not the case as sera from all mice that were tested, made antibody that bound to transfectants expressing HLA-DR; in addition they bound to human B cell lines that did not carry the "tolerizing" HLA molecules (data not shown).

Fusions that were done using the so-called "tolerized" mice and their littermates were again disappointing, as despite a high yield of hybrids, most of the selected hybrids were making monomorphic anti-DR antibodies. However, the limited specificity analysis suggested that one hybrid from R11 fusion was making anti-DR4, which was subsequently confirmed (4, CHAPTER 6). At this point it was

difficult to determine the problem as a number of different immunization schedules and immunogens had been used and a significant number of hybrids grew from each fusion. Retrospectively, it was shown that some of the transfectants used for immunization were infected with mycoplasma; thus some of the hybrids that gave polymorphic, but inconsistent patterns may have been related to mycoplasma-specific antibodies.

The fusions that were done to compare immunization schedules generally yielded a large number of hybrids that were specific for some subtypes of DR4 - molecules. The best fusions came from the group that were immunized subcutaneously with the cells emulsified in CFA and followed by an IP boost at 4-7 weeks. Perhaps the clearest finding was that IP immunization followed by IP boost was the worst of all four combinations. This helped explain the failure of the first set of fusions (Table 5.2), which were mostly done by this method.

Unfortunately, the mice were not bled prior to boosting, but the results of post-boost sera suggest that the titers are not necessarily predictive of the number of HLA-specific hybrids. Intuitively, one feels that high titered sera should yield desirable hybrids, but due to the multiplicity of antibodies in serum, this is not necessarily true. One possibility is that the presence of high titered antibody at the time of immunization may serve to downregulate B cells if they bind antigen via Fc receptor and antibody-receptor. Thus, it may be more appropriate to give the booster after the earlier immune response has subsided.

CHAPTER 6

ANALYSIS OF MONOCLONAL ANTIBODIES SPECIFIC FOR UNIQUE AND SHARED DETERMINANTS ON HLA-DR4 MOLECULES[1]

6.0 ABSTRACT

The specificities for seven monoclonal antibodies to HLA-DR4 were determined using homozygous B cell lines and L-cell transfectants expressing wild-type DR molecules. Three (NFLD.D1, NFLD.M1 and NFLD.D7) bound all DR4 molecules, but only one was specific for DR4. Four antibodies (NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10) reacted with some DR4 subtypes and had extra reactions, particularly, with DR gene products associated with susceptibility to rheumatoid arthritis (RA). To localize the antibody binding epitopes on DR4 molecules, the antibodies were analysed on transfectants expressing hybrid genes, which were generated by exon shuffling of DRB1*0403 and DRB1*0701. Two of the pan-DR4 antibodies bound epitopes which require the β_2 domain while the third mapped primarily to the HVR-I region. One antibody to subtypes of DR4 mapped to residues 40-97 on DRβ1*0403 chains. Comparison of reaction patterns with amino acid sequences suggests that the antibodies against subtypes of DR4 are specific primarily for a region containing sequences postulated to determine susceptibility to RA.

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[1] **In Press: Sheila Drover, Robert W Karr, Xin-Ting Fu, and William H Marshall, Human Immunology, 1994**

6.1 INTRODUCTION

The HLA-DR4 specificity currently consists of fifteen DRB1 allelic variants (DRB1*0401 to DRB1*0415) that have been identified using DNA based technology (Bodmer et al., 1992; Petersdorf et al., 1992; Stastny and Kimura, 1992; Pile et al., 1992; Tiercy et al., 1993; Zhang et al., 1993). Eight of these can be differentiated by T-cells in a mixed lymphocyte typing procedure; they are Dw4 (0401), Dw10 (0402), Dw13.1 (0403), Dw14.1 (0404), Dw15 (0405), DwKT2 (0406), Dw13.2 (0407), Dw14.2 (0408). When the amino acid sequences for the DR4 β1 chains of the T-cell defined subtypes are compared, it is apparent that they differ from DRB1*0401 by only one to four amino acid residues (Table 6.1). Most of the variability is located in the third hypervariable region (HVR-III) around residues 67-74 and at position 86. Residues 1-66 are the same for all alleles except for DRB1*0406 which has serine substituted for tyrosine at position 37, DRB1*0405 which has serine substituted for aspartic acid at position 57 and DRB1*0415 which has glutamic acid substituted for alanine at position 58. The latter DR4 allele (0415), described by Tiercy et al. (1993), differs from the other DR4 alleles in that it has sequence homology with DRB1*1104 at amino acid residues 58-86.

Four alleles DRB1*0401, 0404, 0408 and 0405 which have conservative substitutions in the HVR-III are associated with susceptibility to the development of Rheumatoid Arthritis (RA) in several ethnic groups (Wordsworth et al., 1992).

Table 6.1. Comparison of amino acid differences in DR4 β1 chains of T cell-defined subtypes

DRB1* Alleles	Subtype	Amino acid positions						
		37	57	67	70	71	74	86
B1*0401	**Dw4**	**Y**	**D**	**L**	**Q**	**K**	**A**	**G**
B1*0402	**Dw10**	-	-	**I**	**D**	**E**	-	**V**
B1*0403	Dw13.1	-	-	-	-	**R**	**E**	**V**
B1*0407	Dw13.2	-	-	-	-	**R**	**E**	-
B1*0404	Dw14.1	-	-	-	-	**R**	-	**V**
B1*0408	Dw14.2	-	-	-	-	R	-	-
B1*0405	Dw15	-	S	-	-	R	-	-
B1*0406	DwKT2	S	-	-	-	R	E	V

The fact that other variants (DRB1*0402, 0403, 0406 and 0407) have both non-conservative and conservative substitutions in this region and are not associated with RA susceptibility, has led to the suggestion that a molecular structure resulting from the amino acid sequence 67-86 may play a key role in RA susceptibility (Gregersen et al., 1987). Two non-DR4 alleles, DRB1*0101 and DRB1*1402, which have major differences in HVR-I and HVR-II from each other and from DR4, are completely homologous in HVR-III to each other and to DRB1*0405 and DRB1*0408 (Wordsworth and Bell, 1992; Willkens et al., 1991; Nelson et al., 1992). That these molecules are also associated with RA in different ethnic groups further supports the idea that shared residues (67-86) found on DR4 and non-DR4 β1 chains are somehow critical. However, the mechanism by which this susceptibility is conferred remains unknown.

In the course of attempts to make monoclonal antibodies to the variants of DR4, seven hybridomas whose specificities are the subject of this report were produced; three recognize all DR4 molecules and the other four recognize some of the DR4 subtypes but not others (**CHAPTER 5**). Here we describe a detailed analysis of the specificities of these antibodies using a collection of homozygous B cell lines (BCL) which have well-defined HLA specificities (Yank et al., 1989; Kimura et al., 1992). Mouse L cell transfectants expressing wild type DRB genes were used to confirm the specificity and to identify the DRβ chain responsible for the mAb binding epitope in each case. In addition transfectants expressing

DR(β1*0403/β1*0701) hybrid molecules were used to locate the primary sequences on the DR4 molecule that are essential for binding each of these mAbs.

6.2 MATERIALS AND METHODS

6.2.1 Cells

Homozygous BCL, obtained through the 10th International Histocompatibility Workshop (IHW) were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS), 50units/ml penicillin and 50μg/ml streptomycin, (P/S), and 2mM sodium pyruvate, (Flow Laboratories). Transfectants were cells of the DAP.3 subclone of class II negative murine L cell fibroblasts which had been transfected with DRA and DRB cDNA constructs as described previously (Klohe et al., 1988). The source of the cDNAs and a list of the transfectants, which includes some of those distributed by the organizers of the 11th IHW, are shown in Table 6.2. Construction of DR4 (B1*0403) and DR7 (B1*0701) hybrid cDNAs, by the hemidomain shuffling method and subsequent transfection, has been described (Alber et al.,1989; Fu et al., 1992). All transfectants were grown in Dulbecco's modified Eagles medium containing 10% FBS, 5 x 10^{-5} mM 2-mercaptoethanol and P/S. The cells were grown on either 10 cm dishes (Falcon) or 75cm flasks (Linbro) and were harvested in log phase using 0.25% trypsin (Flow Laboratories) in phosphate buffered saline.

Table 6. 2. HLA-DR expressing transfectants used for characterizing the specificities of NFLD monoclonal antibodies.

Cell Line	HLA-DR Dw Specificities	Allele	11th IHW Number	Contributor
DAP-3 DR1	DR1 Dw1	DRB1*0101	8103	R. Sekaly/ E. Long
DR-BON	DR Bon	DRB1*0103	8104	R. Claude/ C. Thomsen
L466.1	DR15 Dw2b	DRB1*1501	-	R. Karr
L468.5	DR15 Dw12b	DRB1*1502	-	R. Karr
L415.2	DR16 Dw21b	DRB1*1601	-	R. Karr
L168.2	DR3 Dw3	DRB1*0301	8112	R. Karr
DAP-3 DR4	DR4 Dw4	DRB1*0401	8115	R. Sekaly/ E.Long
L243.6	DR4 Dw4	DRB1*0401	-	R. Karr
L164.11	DR4 Dw10	DRB1*0402	8116	R. Karr
L89.2	DR4 DwTAS	DRB1*0403	8123	R. Karr
L165.6	DR4 Dw14	DRB1*0404	8118	R. Karr
L566 H6	DR4 Dw15	DRB1*0405	-	R. Karr
B18	DR4 DwKT2	DRB1*0406	8122	R. Lechler
L91.7	DR11 Dw5	DRB1*1101	8125	R. Karr
L167.2	DR14 Dw9	DRB1*1401	8126	R. Karr
L182.1	DR14 Dw16	DRB1*1402	8127	R. Karr

Table 6.2 Continued.

Cell Line	HLA-DR Dw Specificities	Allele	11th IHW Number	Contributor
L605.1	DR7 Dw17	DRB1*0701	-	R. Karr
29.0.C.27	DR10	DRB1*1001	8131	H. Peter
L416.3	DR51(DR15) Dw2a	DRB5*0101	-	R. Karr
L467.1	DR51(DR15) Dw12a	DRB5*0102	-	R. Karr
L414.1	DR51(DR16) Dw21a	DRB5*0201	-	R. Karr
L575.1	DR52a Dw24	DRB3*0101	-	R. Karr
L105.1	DR52a Dw25	DRB3*0102	8134	R. Karr
L576.5	DR52c Dw26	DRB3*0103	-	R. Karr
L17.8	DR53	DRB4*0101	8138	R. Karr

6.2.2 Antibodies

Generation of mAbs (NFLD.D1, NFLD.D7, NFLD.D2, NFLD.D8, NFLD.D3 and NFLD.D10) using transfectants as immunogens has been described elsewhere (9); all are IgG1 antibodies. Other mAbs used in these studies include NFLD.M1 which reacts preferentially with DR4 molecules (Drover et al., 1985) and L243 which reacts with a monomorphic determinant on DR molecules (Lampson and Levy, 1980). L243 was obtained as a hybridoma from the American Type Culture Collection. The secondary antibody for CELISA, affinity purified F(ab')2 fragments of goat anti-mouse IgG + IgM (heavy and light chains) conjugated to horse radish peroxidase (GAM-HRP), was obtained from Jackson Immunoresearch Laboratories.

6.2.3 Binding Assays and Data Analysis

Optimally-diluted mAbs were tested against the various cell types using a cellular enzyme linked immunosorbent assay (CELISA) essentially as described for human BCL (Morris et al., 1982; Drover and Marshall, 1986) with the following modification for transfectants. After harvesting, the transfectants were left in bacteriological-type petri dishes, to which they do not adhere, for up to 5 days. For assays, transfectant cells were washed and plated in culture medium at 5 x 10^4 cells per well in a 96-well sterile microtiter tray (Linbro) and left to adhere to the plastic

for a minimum of 2 hours at 37° in a CO_2 incubator. Medium was removed from the wells by flicking; cells were washed once in 0.05% Tween 20 (Sigma) in phosphate buffered saline and assayed as previously described (Drover and Marshall, 1986).

CELISA binding data obtained for test mAbs were analyzed relative to the positive control (L243) as described previously (Drover et al., 1985). Essentially, a percent (%) binding value was obtained as follows: the background optical density (OD) was subtracted from each test and the mean OD value from replicate wells was expressed as a percent of the mean OD value of the reference mAb L243. In order to compare binding of the test mAbs to transfectants expressing DRB1*0403/B1*0701 hybrid molecules, the results were double normalized by dividing the percent binding to the hybrid by the percent binding to the DR(β1*0403) wild type transfectant as follows:

$$\frac{\%\ \text{Binding to DRB1*0403/0701 Transfectant}}{\%\ \text{Binding to DRB1*0403 Wild Type Transfectant}} \times 100$$

6.3 RESULTS

6.3.1 Specificity Analysis of DR4 mAbs

Prior to performing specificity analysis, the mAbs were titered using the appropriate cells. The dilution of L243, the DR reference mAb, that gave an OD of 1.5 to 2.0 was chosen as the positive control. For analysis each test mAb was diluted so that it gave at least 100% binding relative to L243 binding with the immunizing cells. For most of the DR4 mAbs this dilution gave greater than 100% binding with BCL expressing the prototype DR4 gene. The reactivity patterns for the mAbs can be divided into two categories, those that bound all DR4 subtypes and those that bound some of the DR4 subtypes. With the exception of NFLD.D1 which is a pan-DR4 with no extra reactions, each mAb also recognized at least one other DR specificity.

TABLE 6.3. Reactivity of NFLD.D monoclonal antibodies with human B cell lines.

	HLA-CLASS II[b]					Converted CELISA Scores For mAb Binding[c]					
Cell	DR	DR Alleles				D1	D7	D2	D8	D3	D10
Lines[a]	Specificity	B1*	B3*	B4*	B5*						
9034	4,53	0401		0101		8	10	10	10	10	10
9027	4,53	0401		0101		10	10	10	10	10	10
9026	4,53	0402		0101		10	10	1	1	1	1
9030	4,53	0407		0101		10	10	6	6	6	10
9024	4,53	0406		0101		10	10	6	8	6	10
9098	4,53	0404		0101		10	10	10	10	10	10
HAS15	4,53	0405		0101		10	10	10	6	8	10
9002	1	0102				1	2	10	6	6	10
9003	1	0101				1	4	10	10	8	10
9010	15,51	1503			0101	1	10	6	6	10	10
9011	15,51	1502			0102	1	10	8	6	10	10
9009	16,51	1601			02	1	10	10	10	10	10
9016	16,51	1602			02	1	10	10	10	10	10
9023	17,52	0301	0101			1	10	1	1	4	2
9021	18,52	0302	0101			1	10	1	1	1	1
9037	11,52	1101	0202			1	10	1	1	1	1
9038	12,52	1201	0202			1	10	1	1	1	1
9060	13,52	1301	0202			1	10	1	1	1	10

TABLE 6.3 Continued

HLA-CLASS II[b]						Converted CELISA Scores For mAb Binding[c]					
Cell Lines[a]	DR Specificity	DR Alleles B1*	B3*	B4*	B5*	D1	D7	D2	D8	D3	D10
9059	13,52	1302	0301			1	4	1	1	1	1
9055	13,52	1302	0301			1	6	1	1	1	1
9057	14,52	1401	0201			1	10	1	2	1	8
9064	14,52	1402	0101			1	10	10	10	10	10
9049	7,53	07		0101		1	1	1	1	2	1
9047	7,53	07		0101		1	1	1	1	1	2
9096	7,53	0702		0101		1	1	1	1	1	4
9068	8	0801				0	1	0	2	1	1
9071	8	0802				1	1	1	1	1	1
9070	8	0803				1	1	1	1	1	1
9076	9,53	0901		0101		1	1	1	1	1	6

[a] refers to the 10th International Histocompatibility numbers designated for the homozygous B cell lines (reference 10)

[b] the HLA class II types and splits were obtained from references 10-11

[c] the CELISA data converted to serology scores: 1, 0-10% binding; 2, 11-20%; 4, 21-40%; 6, 41-80%; 8, 81-100%; 10, > 100%; 0, not done.

6.3.1A mAbs that react with all DR4 molecules

NFLD.D1 was derived from a fusion in which the mouse was immunized with transfectants expressing DRB1*0403 (**CHAPTER 5**). It reacted positively with DR4 positive BCL (Table 6.3) as well as with DR4 positive peripheral blood B cells (data not shown), but it did not bind any DR4 negative B cells. This pan-DR4 specificity was confirmed using transfectant/s expressing individual wild type DRB genes. (Fig. 6.1a). It is apparent that NFLD.D1 bound all six transfectants expressing individual DR4 variants and did not bind any transfectant expressing other DRβ1 chains, nor any chains derived from DRB3, DRB4 or DRB5 genes. NFLD.D1 is thus specific for a determinant found only on DR4 molecules.

NFLD.M1 was previously shown to bind strongly to DR4 positive BCL and weakly to DR3, DR5, DR6, and DR8 positive BCL (Drover et al., 1985). With transfectants (Fig. 6.1b) this antibody recognized an epitope on all DRB1*04 variants as predicted. NFLD.M1 reacted also with transfectants expressing DRB3 genes (DR52 variants), but failed to react with those expressing other DRB1 genes (0301, 1101, 1401, 1402) which explains its extra reactions with B cell lines; transfectants expressing DRB1*08 genes were not available for testing. There was also weak reactivity with transfectants expressing DRB5*0201.The major reactions of NFLD.M1 were thus with all DR4 molecules and with molecules containing DRβ3 chains.

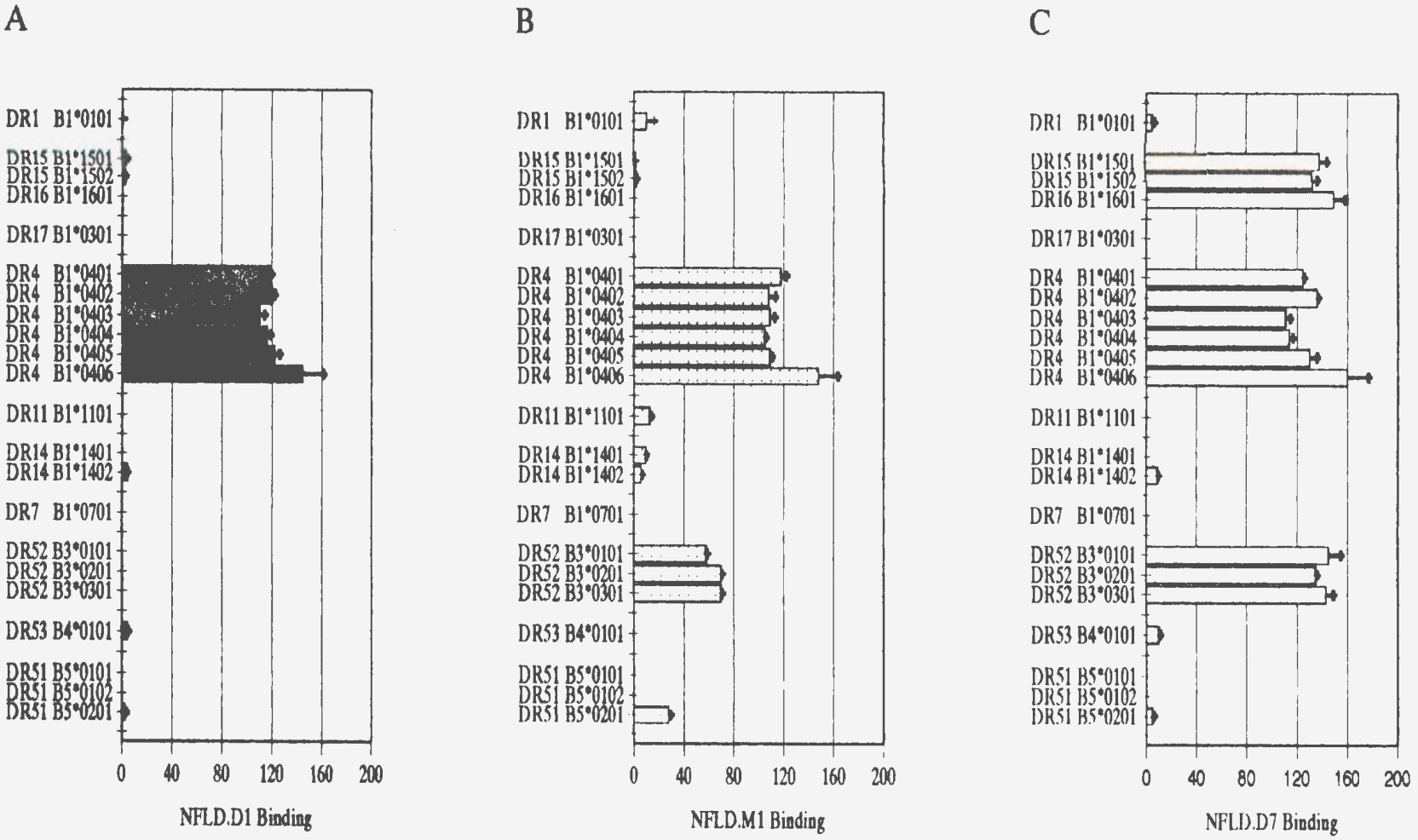


Figure 6.1 Binding of NFLD.D1 (A), NFLD.M1 (B), and NFLD.D7 (C) to mouse cell transfectants expressing DR molecules. The DR specificities and DRB genes expressed by the transfectants are shown to the left of each panel. Binding of each mAb is expressed as a percent of L243 as described under methods. Error bars show the standard error of the mean.

NFLD.D7 was derived from a fusion in which the mouse was immunized with DAP.3 DR4 transfectants expressing DRB1*0401 (**CHAPTER 5**). As can be seen from Table 6.3, NFLD.D7 bound strongly to all DR4, DR15 and DR16 positive BCL. It also bound strongly to most DR52 positive BCL with the exception of two cells, 9055 and 9059, which expresses DRB3*0301. This complex pattern of reactivity was elucidated further by analysis with transfectants. The results presented in Fig. 6.1c clearly show that binding to DR4 positive cells was due to DR4 β1* chains. Binding to DR15 and DR16 cells was due to molecules encoded by DRB1 (1501, 1502,1601) and not by DRB5 genes. The data presented in Table 6.3 showing positive NFLD.D7 binding to most DR52 positive BCL and negative binding to DR8 positive cells (B1*0801, 0802, and 0803), which carry DR52-like determinants, but do not carry DRB3* genes (Table 6.3), suggested that NFLD.D7 might bind molecules encoded by DRB3 genes. As can be seen from the reactivity pattern with transfectants (Fig. 6.1c) this was confirmed; NFLD.D7 only bound DRB3 gene products (DR52) and did not bind the associated DRB1 gene products (DRB1*0301, 1101, 1401 and 1402). NFLD.D7 is therefore reactive with all DR4, DR15 and DR16 and most DR52 molecules.

6.3.1B mAbs that react with a subset of DR4 specificities

NFLD.D2 and NFLD.D8 were derived from the same mouse spleen, while NFLD.D3 and NFLD.D10 were derived from two different mice. In all cases the immunizing cells were DAP.3 DR4 (B1*0401) transfectants (**CHAPTER 5**). The reactivity patterns for all four mAbs with BCL (Table 6.3) were similar, since they reacted strongly with cells expressing some DR4 subtypes DR(B1*0401, 0404, 0405) but did not bind cells expressing DRB1*0402; they differed in the degree to which they bound cells expressing the DR4 subtypes, B1*0407* and B1*0406. It is also apparent from Table 6.3 that these antibodies bound, with varying degrees, to cells expressing variants of DR1, DR15, DR16, and DR14; NFLD.D10 additionally bound to DR9. Since B cells express at least three class II molecules, the precise target of each antibody could not be proven from those data. Transfectants expressing single species of β-chains were useful for this purpose.

Studies using the transfectants confirmed that all four antibodies react strongly with DRB1*0401, 0404, and 0405 and not with DRB1*0402. However, NFLD.D2 and NFLD.D3 (Fig 6.2a and 6.2c) were essentially negative with transfectants expressing DRB1*0403 and 0406 genes, NFLD.D8 (Fig 6.2b) bound weakly, while NFLD.D10 (Fig 6.2d) was strongly reactive with the same transfectants. Binding patterns with products of the relevant non-DR4 genes showed that the majority of the positive reactions of these four antibodies could be attributed to DRB1 chains (Fig. 6.2).

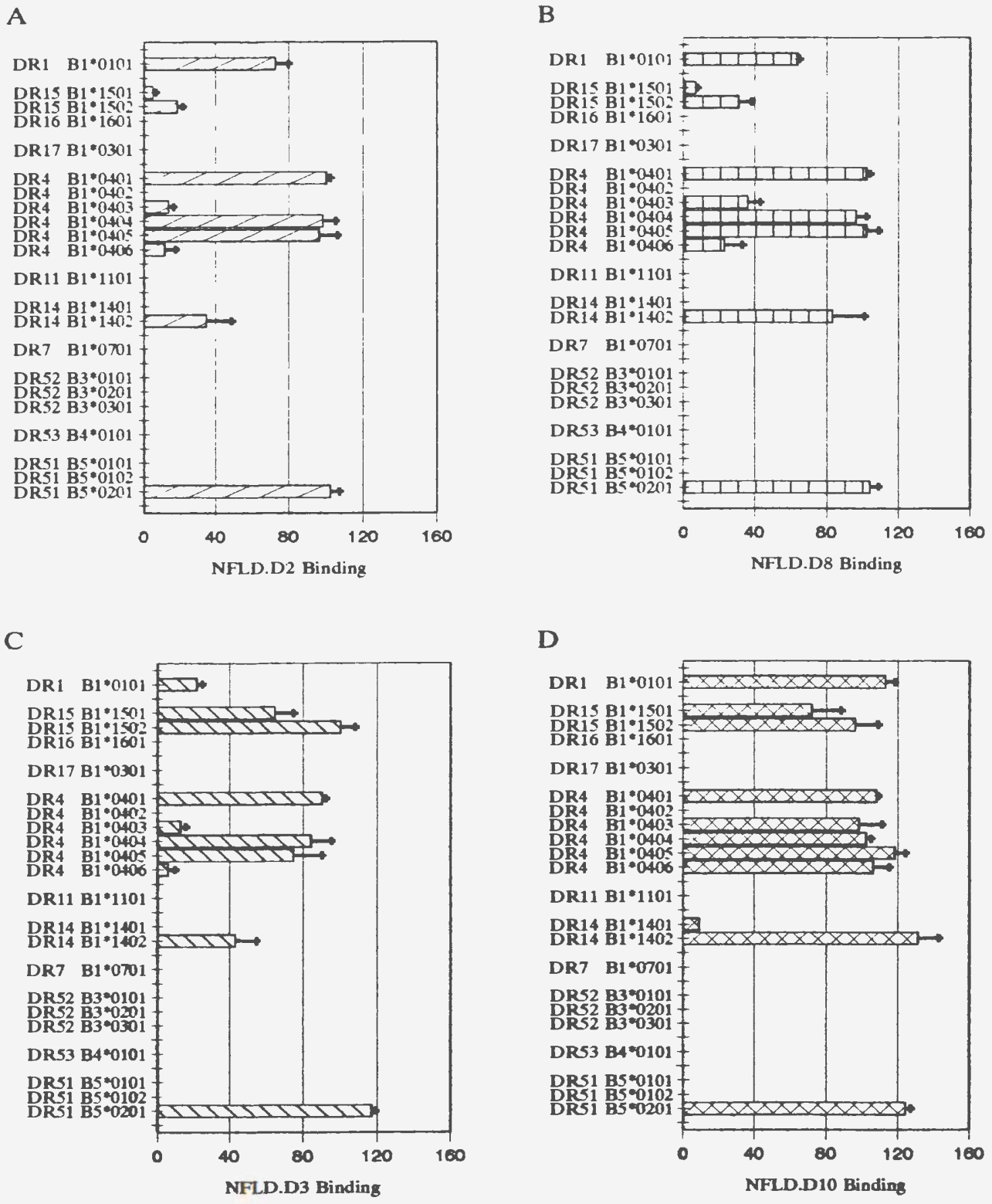


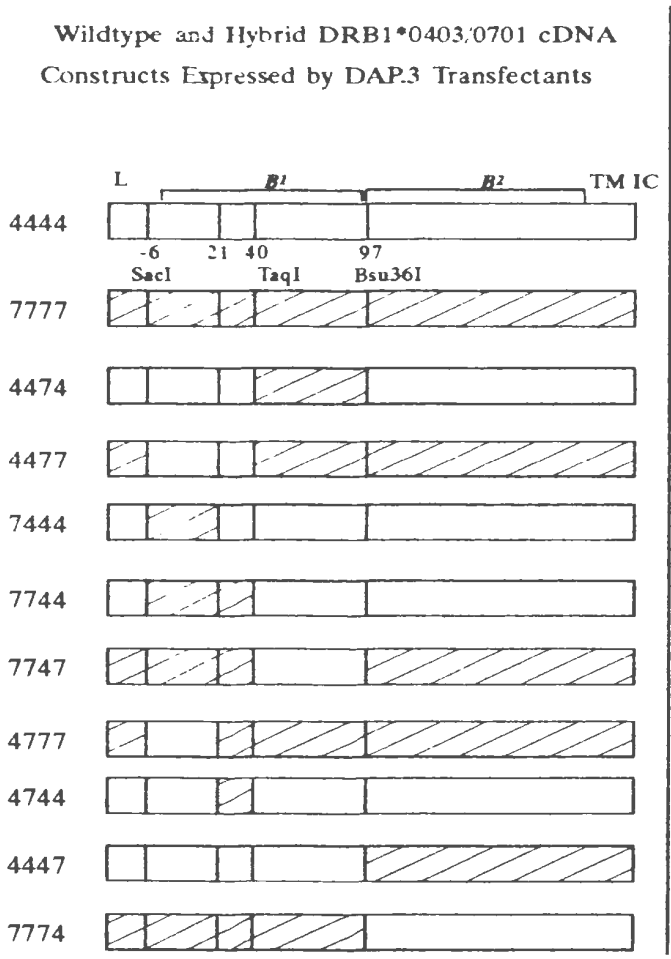
Figure 2. Binding of NFLD.D2 (A), NFLD.D8 (B), NFLD.D3 (C) and NFLD.D10 (D) to transfectants, as described in Figure 1.

One notable exception is that the DR51 transfectant (DRB5*0201) bound all four mAbs, thus identifying DRβ5* as the source of the epitope for all four mAbs in DR16 positive B cells. Transfectants expressing other relevant DRB genes such as DRB4, DRB5*01 and DRB3 did not bind any of these mAbs. It is interesting that the binding patterns of these four antibodies with DR1, DR15, and DR14 transfectants were somewhat different from each other (Fig. 6.2) and that these binding patterns did not always correlate with those obtained with BCL expressing the relevant genes (Fig. 6.4). The possibility that chains from other loci e.g. DQ or DP could bind these mAbs seemed unlikely from an examination of the rest of the BCL data; furthermore, testing on the 11th IHW panel of transfectants expressing DQ and DP was negative (data not shown). Thus, these four antibodies recognize a subset of DR4 molecules and in addition a varying constellation of other DR molecules particularly in the sets coded on DR1, DR14 and DR15 and DR16 haplotypes.

6.3.2 Epitope Mapping Using DRβ1*0403/β1*0701 Hybrid Molecules

Transfectants expressing hybrid DRβ chains have proven useful for locating the critical sequences that determine certain antibody binding epitopes (Alber et al., 1989; Fu et al., 1992). The eight DRB1*0403/DRB1*0701 hybrid cDNAs and the wild type cDNAs used in these mapping experiments are shown in Fig. 6.3. Each hybrid is referred to by four numbers; the first segment represents the leader sequence and is not included in the numbering. The first number refers to amino acid residues -6 to 21, the second to residues 22-40, the third to residues 41-97, and the fourth number refers to the β_2 domain. Antibody binding to the hybrid molecules was measured by CELISA; each antibody was tested in duplicate on two different occasions and the results were double-normalized as described under methods.

NFLD.M1, previously shown to bind an epitope in the second domain of DRβ*0403 molecules, was included as a control; the previous results (Alber et al., 1989) with this antibody were confirmed. NFLD.D1 which is monospecific for DR4 produced a binding pattern remarkably similar to that of NFLD.M1, binding only those hybrids (4474, 7444, 7744, 4744, 7774) that contained the DR4 β_2 domain (Fig. 6.3b). However, NFLD.D1 binding to 4474 and 7774 hybrid molecules was somewhat less than that of NFLD.M1 binding, while its binding to 7444 was increased relative to that of NFLD.M1. Significantly, there was no binding of either antibody to hybrid molecules where DR7 sequences formed the second domain.



NFLD mAb Reactivity with Transfectants Expressing cDNA Constructs

	M1	D1	D7	D10
4444	100	100	100	100
7777	0	0	0	0
4474	145	59	110	0
4477	0	0	125	0
7444	63	124	0	34
7744	145	113	8	130
7747	2	0	9	140
4777	0	0	0	0
4744	111	120	42	106
4447	2	0	100	113
7774	145	87	0	0

Figure 6.3 The panel on the left shows a schematic diagram of the wild type DR(β1*0403) (open bars), DR(β1*0701) (hatched bars) and the recombinant hybrid DR(β1*0403/0701) (open and hatched bars) chains. Each wild type or hybrid β chain is referred by four numbers to indicate the origin of the four segments: first number, amino acid residues -6-21; second number, residues 22-40; third number, residues 41-97; fourth number, β_2 domain. 4 = DR(β1*0403); 7 = DR(β1*0701); L, leader sequence; β_1, first domain; β_2, second domain; TM, transmembrane region; IC, intracytoplasmic region. Sac I, Taq I and Bsu36I in the first drawing refers to the restriction enzyme sites used in the construction of some of the hybrids. The panel on the right shows the degree of mAb binding to the hybrid molecules. Binding has been double-normalized to that obtained for the transfectants expressing DR(β1*0403) wild type molecules, as described under methods.

Because the reaction patterns for NFLD.D1 and NFLD.M1 were different, these data suggest that residues in the second domain form two distinct antibody binding epitopes.

NFLD.D7, which binds an epitope conferred by DRβ1 chains of all DR4, DR15, and DR16 molecules and by DRβ3 chains of DR52 molecules (Fig. 6.1c), showed a complex binding pattern with the hybrid molecules (Fig. 6.3b). It reacted strongly with 4474, 4477, and 4447 molecules, and less but significantly with 4744 hybrid molecules. There was no binding with the 7444, 7744 or 7747 hybrid molecules. This suggests that residues 1-21 on the β1 strand play a major role in the formation of the NFLD.D7 epitope. However, since binding to 4744 hybrid molecules was considerably reduced and there was no binding to 4777, it is likely that residues in other regions of the molecule, particularly HVR-II, also contribute to the integrity of this epitope.

Of the DR4 mAbs that bind a subset of DR4 molecules, only NFLD.D10 was shown to bind strongly to DRβ1*0403 molecules (Fig. 6.2d), and was therefore the only one available for epitope mapping using these particular hybrid molecules. Only transfectants expressing the hybrid molecules that contained the DRB1*0403 sequence 41-97 (7444, 7744, 7747 and 4744) bound this antibody (Fig 6.3b). All other transfectants expressing hybrid molecules (4474, 4477, 4777 and 7774) were negative for NFLD.D10. This indicates that one or more residues near the carboxyl end of the β_1 domain are required for NFLD.D10 binding.

6.4 DISCUSSION

The use of three different types of target cell (B cell lines, transfectants expressing wild-type DR molecules and transfectants expressing hybrid DR4/DR7 molecules) to study these antibodies has allowed considerable progress towards identifying their epitopes. A summary of the specificity analysis resulting from reactions with BCL and transfectants is given in Fig. 6.4. Immunofluorescence and flow cytometry have shown that the antibodies also bind to peripheral blood B cells (unpublished data). All are IgG1 and could not be studied by complement dependent cytotoxicity.

The reactions on B cell lines and on L-cell transfectants expressing the same genes were not always comparable (see Fig. 6.4), despite careful normalization of the assay results to those for the reference antibody, L243. There are many differences between EBV transformed B cell lines and mouse fibroblasts, as hosts for DR genes, that might explain these differences; glycosylation patterns, the amount of invariant chain available in DAP.3 transfectants, peptide processing pathways and the range of available peptides for insertion into the groove may differ. The importance of chaperone-like molecules, such as the invariant chain, and the requirement for peptide to produce the correct folding of class II molecules has been documented (Anderson and Miller, 1992; Sette et al., 1992; Germain and Hendrix, 1991).

SEROGRAM OF ANTI-DR4 MONOCLONAL ANTIBODIES

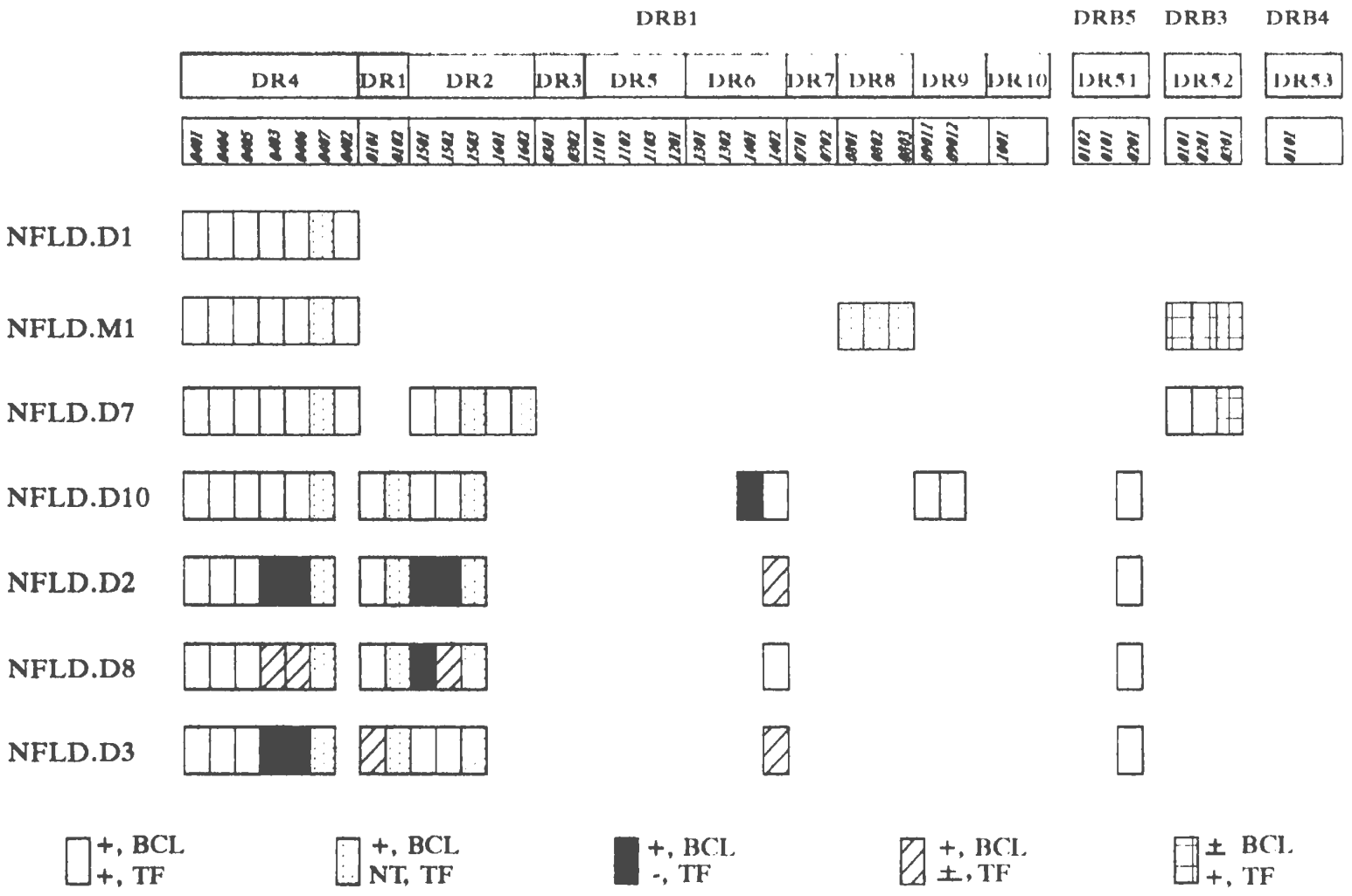


Figure 4. Summarizes anti-DR4 mAb reactivity with BCL and transfectants expressing different HLA-DR specificities. +, positive (>40% reactivity); -, negative (< 20% reactivity); +, weakly reactive (21-40% reactivity); NT = not tested.

It is notable that neither of the antibodies which bound to second domain epitopes showed any reduction of binding to transfectants while the mAbs which recognized subtypic determinants on DR4 molecules did. NFLD.D2 was most affected by the nature of the host cell, which suggests that its binding epitope is more influenced by peptide-in-the-groove than are the binding epitopes of the other antibodies. An extreme example of such an antibody is one we have recently produced (**CHAPTER 8**) that behaves like an alloreactive T cell, recognising the DRβ1 *0401 molecule and no other; this antibody completely fails to bind to transfectants expressing DRB1 *0401. If alloreactive T cell recognition involves co-recognition of residues on the alpha helix plus self peptide, as is currently believed (Lechler and Lombardi, 1991) then it follows that an antibody which can discriminate the same T cell-defined subtypic variant will also be peptide dependent. Such an antibody was also described by Murphy et al (1989).

Considering the paucity of mAbs such as NFLD.D1 which define the DR serotype, it is informative to examine the DR4 β1 chains for the presence of unique sequences. The first possibility is residues 9-13:EQVKH which are located on the floor of the peptide binding groove (Brown et al., 1993). Two other possibilities are single residues located in the β_2 domain, tyrosine (Y) at position 96, which was earlier suggested as the polymorphism defining DR4, (Gorski, 1989) and leucine (L) at position 180 which is near the transmembrane portion of the molecule (Bell et al., 1987). Although the serological pattern for NFLD.D1 (Table 6.3 and Fig. 6.1a)

appears identical to that reported for GS359-13F10 (Alber et al., 1989), analysis with the hybrid molecules indicate that these mAbs bind two disparate epitopes. The GS359-13F10 epitope was initially localized to the region 41-97 (Alber et al., 1989), and was later more definitively mapped to 96Y (Maurer and Gorski, 1991). The possibility that NFLD.D1 might also map to 96Y was examined in relation to the binding patterns obtained with the hybrid molecules (Fig. 6.3). Since it did not bind any transfectants that expressed the DR4 sequence 41-97 unless the DR4 β_2 domain was also present, 96Y is unlikely to be important for NFLD.D1 binding. Even if binding is partly dependent on the sequence YPE (96-98), which is normally found only on wild type DR4 molecules, then NFLD.D1 would be expected to bind the hybrid molecules 7747 and 4447 because DR7 carries E98 and DR4 carries YP (96-97). Thus lack of binding of NFLD.D1 to hybrid molecules carrying YPE in the absence of the DR4 β_2 domain, suggests that other residues in the β_2 domain are critical for NFLD.D1 binding. A possible candidate for the NFLD.D1 epitope is 180L. Experiments are in progress to test this hypothesis.

Attempts to map antibody epitopes on DR hybrid molecules without first identifying the DRβ chain that confers the binding epitope, can lead to errors. For example, our earlier studies using BCL and DR hybrid molecules suggested that the epitope for NFLD.M1 is located in the second domain of DRβ1 chains expressed by DR4, DR3, DR5, DR6 and DR8 positive cells (Alber et al., 1989; Drover et al., 1985). Based on these findings we predicted that the critical residue was Threonine

(T) at position 140 on DR β1 chains. When the analysis was extended, in the present studies, to include transfectants which express individual β1, β3, β4 and β5 chains (Fig 6.1b), NFLD.M1 was found to bind transfectants expressing DRB3*0101, 0201 and 0301 genes, but not to those transfectants expressing DRB1*0301, 1101, 1401 and 1402. Furthermore, comparison of the sequences of DRβ3 chains shows that they do not have 140T; neither do they have any residue in the first or second domain that is unique to DR52 and DR4 molecules. Thus, although we have confirmed that NFLD.M1 binding is critically influenced by residues in the β2 domain of DR4 molecules, the epitope appears more complex than we had earlier anticipated.

A further example of the complexity of antibody binding epitopes is illustrated by NFLD.D7. Its specificity is similar to NFLD.M1 in that it binds DR4 and DR52 molecules; unlike NFLD.M1, it also binds DR15 and DR16 β1 chains. Analysis with the hydrid molecules shows that NFLD.D7 binding is mainly influenced by residues near the amino terminus of the β_1 domain, notably residues 1-21. Comparison of DR sequences does not reveal any residues unique to DR4, DR15, DR16 and DR52 molecules. Furthermore, residues 1-21 which are important for NFLD.D7 are unlikely to be available for antibody binding (Brown et al., 1993). It seems likely then, that NFLD.D7 recognizes a conformational determinant that is dependent on the interaction of residues in HVR-I and HVR-II regions of DR4 molecules. Formation of this epitope may depend on different regions in DR15, DR16, and DR52 molecules.

Epitope mapping with NFLD.D10 showed that the sequence 41-97 in DRB1*0403 is essential for preservation of its epitope (Fig. 6.3b); unfortunately, mapping experiments with the hybrid molecules were not feasible for NFLD.D2, NFLD.D3, and NFLD.D8 since they either do not bind (or bind poorly) to transfectants expressing DRB1*0403. However, the similarities in specificity between these four antibodies when exposed to other DR4 subtypes, both in transfectant and BCL forms, suggest that they too probably bind to an epitope involving the sequence 41-97.

The four antibodies to DR4 subtypes show reaction patterns that parallel, to varying degrees, the presence of a "shared sequence" that is associated with susceptibility to development of RA (Gregersen et al., 1987; Duquesnoy et al., 1984). This sequence at positions 70-74 on the beta chain is QKRAA on DRB1*0401 and QRRAA on the other DR4 alleles that are associated with RA (DRB1* 0404, *0408) and on the DR1 and DR14 alleles that are similarly associated (DRB1*0101,*1402). An analysis using site-directed mutagenesis of relevant DRB genes to map these epitopes at the level of single amino-acid substitutions is underway.

CHAPTER 7

AMINO ACIDS IN THE PEPTIDE-BINDING GROOVE INFLUENCE AN ANTIBODY-DEFINED, DISEASE-ASSOCIATED HLA-DR EPITOPE[1]

7.0 ABSTRACT

A shared amino acid sequence on the alpha helix of certain DRβ1 chains is predicted to generate a "shared epitope" that is implicated in susceptibility to the development of rheumatoid arthritis (RA). Different relative risks (RR) for disease susceptibility and severity conferred by these DRβ1 chains suggest that their "shared epitopes" are not equivalent. A set of monoclonal antibodies that map to the critical region, and for which optimal binding depends on DR context and cell lineage, was used to test this idea. Mapping experiments using mutated DRβ1* molecules showed that the antibody-binding epitopes are overlapping; residue 70Q is pivotal for each, but neighbouring residues on the alpha helix and on the floor of the groove are also involved. Importantly, these epitopes are profoundly modified by peptide loading of DRβ1*0401 molecules. These data suggest that "shared epitopes" on DR molecules that are associated with RA are influenced by their context; such structural modifications may be the basis for the varying susceptibilities conferred by these DR molecules for the development of RA.

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[1]**In Press: Sheila Drover, William H Marshall, William W Kwok, Gerald T Nepom, Robert W Karr, Scandinavian J Immunology, 1994**

7.1 INTRODUCTION

HLA molecules, which are encoded by the major histocompatibility complex (MHC), are important for binding and presentation of antigenic peptides to T lymphocytes. These HLA molecules show extensive polymorphism and in some cases are associated with susceptibility to autoimmune diseases (Nepom and Erlich, 1991). HLA-DR4 was found to confer risk (Stastny, 1978) for the development of Rheumatoid Arthritis (RA). However, this risk was not absolute and in some studies in which the prevalence of DR4 was low, DR1 was shown to confer susceptibility (Stastny, 1980). Finer definition of the HLA alleles using T cell typing and molecular methods showed that the DR4 specificity was composed of a family of alleles (DR4-Dw variants) whose risk for RA varied considerably (Zoschke and Segall, 1986; Nepom et al., 1986).

A unifying hypothesis that became known as "the shared epitope hypothesis" (Gregersen et al., 1987) emerged from the discovery of a shared sequence 70-74:QKRAA/QRRAA on the DRβ1 chains of DR4 and DR1 molecules and a similar sequence (RRRAA) on DR10 molecules (Gregersen et al., 1986; Bell et al., 1985; Winchester and Gregersen, 1988; Merryman et al., 1988). Importantly, DRβ1 chains which have one or more non-conservative substitutions in this region, including those contained in the same DR4 context (eg. DRβ1*0402 and 0403), are associated with resistance to the disease (Winchester and Gregersen, 1988; Amar et

al., 1982; Wordsworth et al., 1989). Further evidence for this hypothesis came from the finding that a rare DR6 allele (DRB1*1402), which carries the same shared sequence, is strongly associated with RA in two North American Indian populations (Willkens et al., 1991; Nelson et al., 1992).

The presence of a "susceptibility sequence" in approximately 90% of RA patients of Caucasoid origin (Nepom and Erlich, 1991; Wordsworth et al., 1989; Wallin et al.,1991), implies that the resulting structures on the expressed DR molecules play an influential role in the development of RA. It has been proposed that structures formed by these shared sequences might be conformationally equivalent (Winchester and Gregersen, 1988); yet the relative risk (RR) for developing severe RA is significantly higher when the shared sequence is present on DR4 molecules than when it is present on other DR molecules (Wordsworth et al., 1992; Weyand et al., 1992). Analysis of the published sequences (Gregersen et al., 1986; Bell et al., 1985; Merryman et al., 1988; Marsh and Bodmer, 1992) shows that amino acid residues on the beta strands that make up the floor of the grooves of DR4, DR1, DR14 and DR10 molecules are quite dissimilar. Thus, the varying degrees of risk and severity conferred by these molecules may be related to differences in DR context, peptide binding or orientation, and/ or subsequent conformational alterations to their "shared epitopes". By increasing our understanding of the topography of these structures, some insight into their role in RA and other autoimmune diseases may be gained.

We have previously shown that four mAbs (NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10) have similar specificities and that they bind specifically to those DR molecules associated with RA (**CHAPTER 6**). In the present study, we have extended previous work which suggested that the integrity of these epitopes on the relevant DR molecules is affected by their DR context and by the cells on which they are expressed. Epitope mapping studies for each of the antibodies were performed using a series of DRβ1 mutants produced by site-directed mutagenesis of the relevant DRB1 cDNA. Finally, the contribution of bound peptide in the formation of these epitopes was addressed using an antigen-processing mutant that had been transfected with DRA and DRB1*0401 genes. The combined results from these studies indicate that amino acid substitutions at positions predicted to be involved in peptide binding, and/or changes in peptide processing and presentation, alter the structure of the "shared epitope" that is associated with the development of RA.

7.2 MATERIALS AND METHODS

7.2.1 Cells

Homozygous human B cell lines (BCL) obtained through the 10th International Workshop (IHW) and the DRB genes that they express are as follows: WT51, DRB1*0401 and B4*0101 MT14, DRB1*0404 and B4*0101; KAS116, DRB1*0101; AMALA, DRB1*1402 and B3*0101; SCHU, DRB1*1501 and B5*0101; MGAR, DRB1*1501 and B5*0101; E4181324, DRB1*1502 and B5*0102; KAS011, DRB1*1601 and B5*0201 (CHAPTER 6). The cell line HAS15 (DRB1*0405 and B4*0101) was kindly provided by H. Mervart through the 9th IHW (Gross-Wilde et al., 1984). Mouse cell transfectants were cells of the DAP.3 subclone of class II-negative murine L cell fibroblasts, which had been transfected with DRα and DRβ cDNA, and have been described elsewhere (CHAPTER 6, Inoka et al., 1992). They include L243.6, DRB1*0401; L165.6, DRB1*0404; L566.H6, DRB1*0405; DAP3-DR1, DRB1*0101; L182.1, DRB1*1402; L466.1, DRB1*1501; L468.5, DRB1*1502; L414.1, DRB5*0201; L164.11, DRB1*0402; L168.2, DRB1*0301 and L91.7, DRB1*1101. The T2 mutant (Riberdy and Cresswell, 1992) expressing DRB1*0401 (T2-Dw4) was made in GT Nepom's laboratory (unpublished data). Human BCL were maintained in RPMI-1640 containing 10% heat inactivated fetal bovine serum, 2mM sodium pyruvate, 50 units/ml penicillin and 50μg/ml streptomycin (all obtained

from Gibco, Grand Island, NY). Mouse cell transfectants were maintained in DMEM (Gibco) containing the same supplements plus 5 x 10^5M 2-mercaptoethanol (Sigma, St. Louis, MO).

7.2.2 Generation of DRB1* Mutants

The DRB1*0401 mutants used for epitope mapping were derived by site-directed mutagenesis of DRβ cDNA as previously described (Karr et al., 1990). In each mutant, a single amino acid residue found in the wild type DRβ1*0401 chain was replaced by the residue found at the same position of another DRB1 allele. Thus, positions 37, 57, 67, 70, 71, 74 and 86 were substituted using residues from other DR4 subtypes; another DRB1*0401 mutant was made with residue 70R substituted from DRB1*1001. Positions 9, 11, 13, 28, and 30, which are conserved among the DR4 subtypes, were substituted with residues from DRB1*0701. In a reverse approach several wild type genes, whose products do not bind the antibodies, were mutated at critical points in attempts to create epitopes for these antibodies. Thus, mutants with the following substitutions were generated: DRB1*0402 (70D→Q); DRB1*1101 (70D→Q); DRB1*1101 (70D→R) and DRB1*0301 (73G→A). Transfectants expressing class II molecules were isolated and maintained as previously described (Klohe et al., 1988).

7.2.3 Antibodies

The mAbs NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10 are murine monoclonal antibodies made using L cell transfectants as immunogens, and were previously shown to have similar binding patterns with DR4 variants: β1*0401, 0404 or 0405 (Drover et al., 1992). Control mAbs used to show that the overall integrity of the mutated DRβ1*0401 molecules was not drastically altered, included the following: SG520 (obtained from Sanna Goyert, North Shore University Hospital, Manhasset, NY) and GSP 4.1 (obtained through the 10th IHW) which bind monomorphic determinants on DR molecules (Goyert and Silver, 1981; Knowles et al., 1989); 50D6 and 21r5 (obtained from Michelle Letarte, Hospital for Sick Children, Toronto, Canada), which bind all DR molecules except DR7 (Letarte et al., 1985; Addis et al., 1982), and map to regions 1-40 and 41-97, respectively, on the β1 chains (Addis et al., 1982; Alber et al., 1989); 35913F10 (obtained from Susan Radka, Oncogen, Seattle, WA) and NFLD.D1, which bind all DR4 molecules (Alber et al., 1989; Drover et al., 1992) and map to different regions of the β_2 domain (Maurer and Gorski, 1991;**CHAPTER 6**). L243, obtained from ATCC, binds a monomorphic determinant on all DR molecules that is dependent on DR α chain sequences (Lampson and levy, 1980; Fu and Karr, 1994) and was used as a reference control for DR expression in all these experiments. Affinity purified F(ab')2 fragments of goat anti-mouse IgG + IgM (heavy and light chains), which were

conjugated to horse radish peroxidase (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), were used as the secondary antibody for CELISA. The secondary antibody used for immunofluorescence and flow cytometry was fluoresceinated goat anti-mouse IgG (Organon Technika-Cappel, Malvern, PA).

7.2.4 Binding Assays and Data Analysis

Antibodies were tested in duplicate, usually on two to three different occasions using either a live cell enzyme-linked immunoassay (CELISA) or immunofluorescence and flow cytometry as previously described (Klohe et al., 1988; Drover and Marshall, 1986). Various concentrations of mAbs, culture medium or irrelevant mouse IgG (negative control), and optimally-diluted L243 (anti-DR positive control), were tested on BCL and on transfectants expressing either wild type or mutated DR molecules. To reduce any artifacts that might result from differences in the level of DR expression on these cells, CELISA data on the test mAbs were analyzed relative to the positive control, L243. Essentially, a percent (%) binding value was obtained as follows: the background optical density (OD) was subtracted from each test OD and the mean OD value from replicate samples was expressed as a percent of the mean OD value for L243.

For some of the experiments the data were double-normalized as follows:

$$\frac{\text{OD test mAb on non-DR}\beta\text{1*0401 or mutant - background OD/ OD reference mAb on non-DR}\beta\text{1*0401 or mutant - background}}{\text{OD test mAb on DR}\beta\text{1*0401 - background OD/ OD reference mAb on DR}\beta\text{1*0401 - background}}$$

Binding assays done using immunofluorescence were analyzed on a Coulter Epics 753 flow cytometer (Coulter Cytometry, Hialeah, FL) to determine the mean channel fluorescence (MCF) of each sample. The mean fluorescence (MF) was calculated from the MCF by the formula $MF = 10^{MCL/85.33}$. The data were double normalized using the same formula as shown above, but with MF substituted for OD values.

7.2.5 Statistical Analysis

The significance of differences in mAb binding to relevant DR molecules expressed on BCL and mouse cell transfectants was determined by non parametric (Wilcoxan) analysis of the paired data. To determine if mAb binding to transfectants expressing mutated DRβ1*0401 molecules was significantly different from that obtained for the transfectant expressing wild type DRβ1*0401 molecules, a one way

analysis of variance (ANOVA) was performed; corrected Bonferroni p values (Bailar and Mosteller, 1992) were obtained. Both statistical methods were performed using a computer program called INSTAT (Graphpad Intuitive Software for Science, San Diego, CA).

7.3 RESULTS

7.3.1 The Topography of Shared Epitopes on DR Molecules Differs with Cell Lineage

Previous work, which is summarised in Table 7.1, suggested that the antibody binding epitopes for NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10 were determined primarily by the amino acid sequence 70-74:QKRAA/QRRAA on the HLA-DRβ1 chains that are predicted to carry the putative RA shared epitope. There were also extra reactions including binding to BCL expressing DR15 and DR16 haplotypes which are not associated with RA (Nelson et al., 1992; Ollier and Thompson, 1992). It was subsequently shown, using mouse cell transfectants expressing a single DR specificity, that these mAbs bind molecules carrying DRβ1*15 or DRβ5*02 chains that contain the sequence 70-74:QARAA; they did not, however, bind transfectants expressing DRβ1*16 or DRβ5*01 chains that contain the sequence 70-74:DRRAA (Table 7.1 and CHAPTER 6).

Table 7.1. Comparison of β_1 domain sequences[a] of HLA-DR beta chains, their association with susceptibility to rheumatoid arthritis (RA)[b] and the relative mAb binding[c] to BCL expressing these alleles.

HLA-DR Specificity		Amino acids in the β1 domain																								NFLD.mAb binding				
Sero-type	Allele	9	10	11	12	13	26	28	30	31	32	33	37	47	57	58	67	70	71	72	73	74	77	85	86	RA	D10	D8	D3	D2
DR4	B1*0401	E	Q	V	K	H	F	D	Y	F	Y	H	Y	Y	D	A	L	Q	K	R	A	A	T	V	G	+	++	++	++	++
DR4	B1*0404	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	V	+	++	++	++	++
DR4	B1*0405	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	R	-	-	-	-	-	-	+	++	++	++	++
DR1	B1*0101	W	-	L	-	F	L	E	C	I	-	N	S	-	-	-	-	-	R	-	-	-	-	-	-	+	++	++	+	++
DR14	B1*1402	-	Y	S	T	S	-	E	-	-	H	N	N	-	-	-	-	-	R	-	-	-	-	-	-	+	++	++	++	++
DR14	B1*1401	-	Y	S	T	S	-	D	-	-	H	N	F	-	A	-	-	R	R	-	-	E	-	-	V	0/+	+	-	-	-
DR2	B5*0201	Q	-	D	-	Y	-	H	G	I	-	N	N	-	-	-	I	-	A	-	-	-	-	A	V	0/-	++	++	++	++
DR2	B1*1501	W	-	P	-	R	-	-	-	-	-	N	S	F	-	-	I	-	A	-	-	-	-	-	V	0/-	++	+	++	+/-
DR2	B1*1502	W	-	P	-	R	-	-	-	-	-	N	S	F	-	-	I	-	A	-	-	-	-	-	-	0/-	++	+	++	+
DR4	B1*0403	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	V	0/-	++	+	+/-	+/-
DR3	B1*0301	-	Y	S	T	S	Y	-	-	-	H	N	N	F	-	-		-	-	-	G	R	N	-	V	0/+	-	-	-	
DR4	B1*0402	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	D	E	-	-	-	-	-	V	-	-	-	-	-
DR11	B1*1101	-	Y	S	T	S	-	-	-	-	-	N	-	F	-	E	F	D	R	-	-	-	-	-	-	0/-	-	-	-	-
DR7	B1*0701	W	-	G	-	Y	-	F	L	-	-	N	F	-	V	-	I	D	R	-	G	Q	-	-	-	0/-	-	-	-	-

[a] amino acid sequences, given in the one letter code, were derived from Marsh and Bodmer, (1992).

[b] Rheumatoid arthritis association with HLA-DR alleles: +, positive; -, negative; 0, neutral (references [1])

[c] mAb binding is referenced to L243 as described in methods; binding is scored as follows: -, <25% binding; ±, 26-50%; +, 51-75%; ++, 76-100%.

[1] Associations are described in CHAPTER 2, Table 2.1

An interesting observation was that the antibody binding patterns differed according to whether the DR molecules were expressed on BCL or mouse cell transfectants.

To further analyze the phenomenon of differential binding to BCL and transfectants expressing the relevant DR genes, parallel studies (CELISA) were performed using three of the mAbs, NFLD.D2, NFLD.D3 and NFLD.D10. Statistical analysis of the paired data for each mAb (Table 7.2) revealed highly significant differences in NFLD.D2 binding to BCL and transfectants. The greatest contrast occurred for paired cells expressing DRB1*0101, 1402, 1501, and 1502. Differences in NFLD.D3 binding to BCL and transfectants were marginally significant due mainly to dissimilar binding to paired cells expressing DRB*0101 and 1501. NFLD.D10 bound similarly to both cell types. The most conspicuous differences between mAb binding to BCL and to transfectants were revealed when binding obtained for each on-DRB1*0401 cell was double normalized to binding obtained for DRB1*0401. Paired data for each mAb are shown in Fig. 7.1. The salient feature is that NFLD.D2 reacted strongly with DRβ1*1402 expressing-BCL, but not with the relevant transfectant, while NFLD.D3 and NFLD.D10 reacted equally well or better with this transfectant than with the BCL. In addition, weak NFLD.D2 reactivity with DRβ1*1501 molecules on BCL vanished on the transfectants.

Table 7.2. Comparison of mAb binding[a] to DR molecules expressed on B cell lines (BCL) and mouse cell transfectants (TFS).

HLA-DR[b]	NFLD.D2		NFLD.D3		NFLD.D10	
	BCL	TFS	BCL	TFS	BCL	TFS
B1*0401	126 ± 5. 5[c]	101 ± 1.8	113 ± 3.4	96 ± 2.7	125 ± 4.8	122 ± 1.8
B1*0404	121 ± 7.5	82 ± 0.95	111 ± 7.4	93 ± 3.1	126 ± 9.5	132 ± 4.3
B1*0405	88 ± 5.4	94 ± 3.6	86 ± 2.3	98 ± 2.4	92 ± 1.9	126 ± 7.4
B1*0101	105 ± 5.4	51 ± 1.3	76 ± 3.8	47 ± 1.9	124 ± 2.5	119 ± 2.5
B1*1402	136 ± 6.4	14 ± 4.8	112 ± 0.9	91 ± 5.5	139 ± 9.0	163 ± 5.4
B5*0201	117 ± 6.6	111 ± 1.4	111 ± 7.3	114 ± 3.7	120 ± 7.3	129 ± 6.3
B1*1501	30 ± 0.9	2.2 ± 2.2	85 ± 2.7	55 ± 3.4	116 ± 5.1	88 ± 6.2
B1*1502	86 ± 6.4	21 ± 1.9	103 ± 4.1	98 ± 0.96	133 ± 0	126 ± 4.3
Wilcoxan Test	$p^d = 0.0156$		$p = 0.0547$		$p = 0.6406$	

[a] mAb binding measured in CELISA and normalized to the reference DR control, L243, as described in methods.
[b] DRB alleles expressed by transfectants and BCL.[c] mean of replicate samples ± standard error of the mean.
[d] two tailed p values.

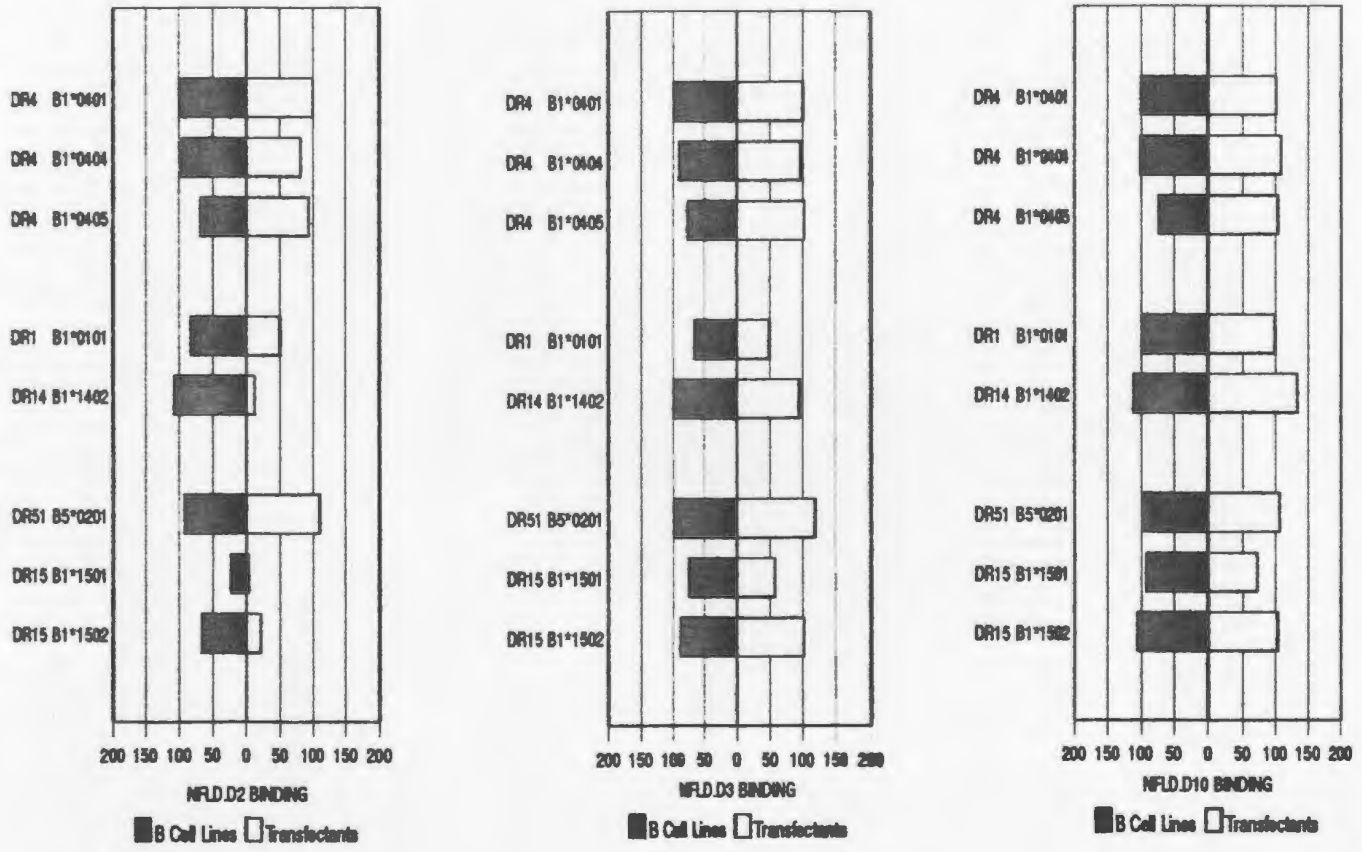

Figure 7.1. Comparison of mAb binding to DR molecules expressed on BCL with that obtained for mouse cell transfectants expressing the prototype genes. The mean relative binding of each mAb to non-DRβ1*0401 expressing cells was double normalized to the mean relative binding to DRβ1*0401 positive cells for both BCL and transfectants, as described under methods.

Other points to note are: i) all mAbs bind more avidly to DRβ1*0405 and DRβ5*0201 when the genes are expressed in transfectants, while all mAbs bind DRβ1*1501 molecules more avidly when they are expressed in BCL; ii) NFLD.D2 and NFLD.D3 binding to DRβ1*0101 molecules, which were somewhat reduced on BCL, was significantly reduced on transfectants; iii) all three mAbs bind differently to cells expressing DRβ1*1502 molecules: NFLD.D2 binds poorly, but more avidly, to BCL than to transfectants; NFLD.D3 binds better to transfectants, while NFLD.D10 binds equally well to both. Taken together these data show that epitopes generated by shared sequences vary according to both the structure of the rest of the DR molecule and to the cell type on which the molecule is expressed.

7.3.2 Contribution of Residues on the Alpha Helix and on the Floor of the Peptide Binding Site to "Shared Epitopes" on the Alpha Helix of DRβ1*0401 Molecules

Comparison of the reactivity patterns for these mAbs with the available sequence data, suggested that the putative RA susceptibility sequence (70-74) is influential in forming these epitopes, and that 70Q may be critical (Table 7.1). However, since these mAbs do not bind all DR molecules carrying 70Q and since they show a gradient of reactivity with DR molecules containing the putative RA

susceptibility epitope, it was predicted that other amino acids, including peptide binding residues might be involved in formation of these epitopes. To test this hypothesis binding was analyzed on a panel of DRβ1*0401 mutants, each containing a single amino acid substitution (Fig. 7.2A), including non-conservative substitutions (70Q→D, 71K→E, 74A→E, 57D→S, 37Y→F 30Y→L, 13H→Y and 9E→W) and conservative substitutions (86G→V, 67L→I, 71K→R, 28D→E and 11V→G).

To ensure that the structural integrity of the DR$\alpha\beta$1*0401 mutated molecules had been maintained, the mutants were analyzed with several well-characterized mAbs. Binding of the two monomorphic mAbs, GSP 4.1 and SG520, to the mutant molecules were identical to that obtained for wild type molecules (data not shown). The DR4-specific mAbs (359 13F10 and NFLD.D1), which map to different regions of β_2 domain and two mAbs (50D6 and 21r5), which map to different regions of the β_1 domain showed no significant variation in binding to mutant molecules from that observed for wild type molecules (Fig. 7.2B). These data suggested that the overall structural integrity of the mutated DRβ1*0401 molecules had been maintained, thus permitting confident analysis of the following data on antibody binding epitopes.

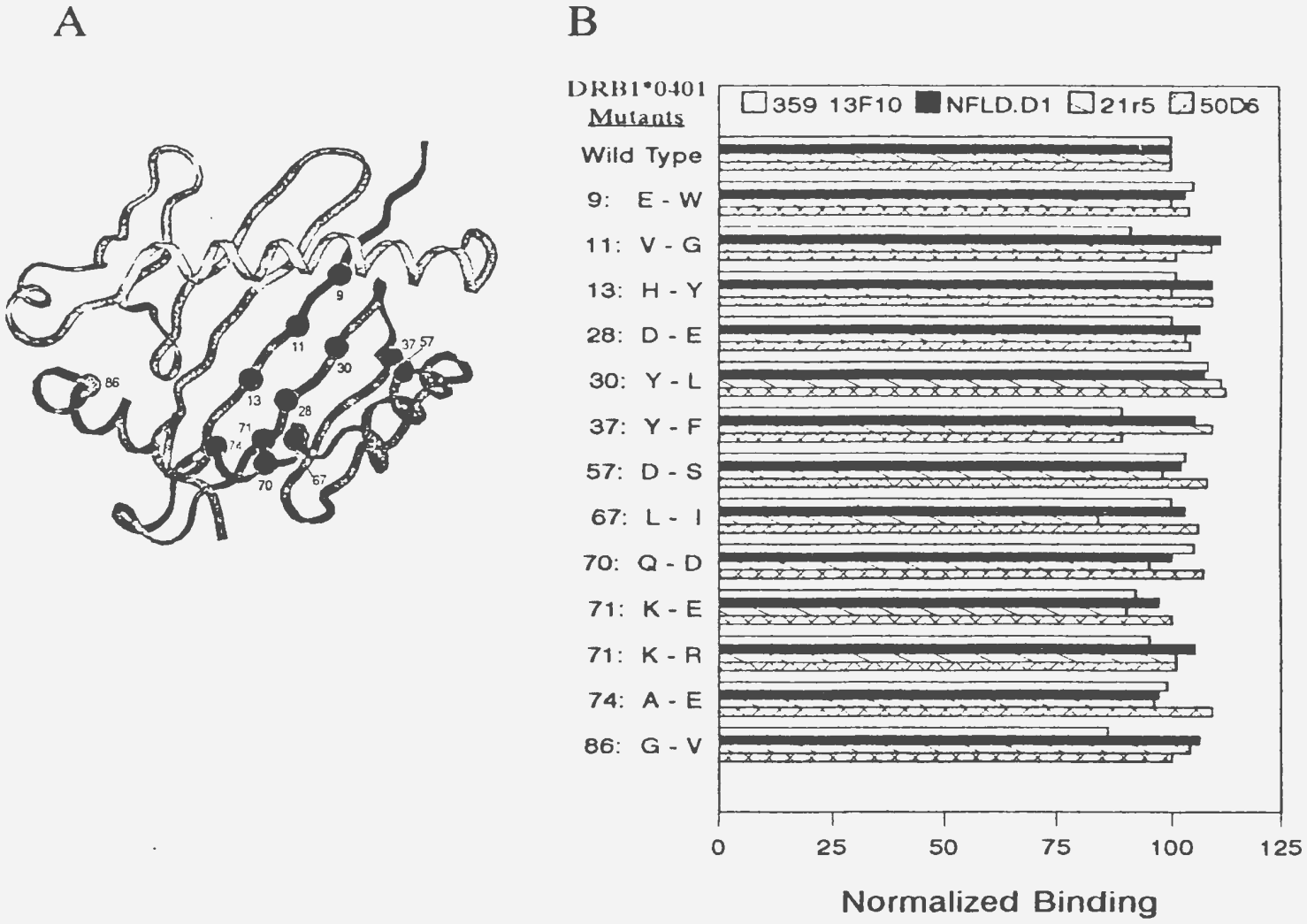

Figure 7.2. A) Schematic representation of the class II peptide binding site. The coordinates for the structure of HLA-DR1 (Brown et al., 1993) were provided by Jerry Brown, Harvard University, and the structure was viewed on an IRIS4D/310GTX workstation (Silicon Graphics, Mountain View, CA) using Insight II software (Biosym Technologies, San Diego, CA). The location of the amino acid substitutions created in the mutants of DRB1*0401 are shown as circles. B) Analysis of the conformational integrity of the mutated DRB1*0401 with a panel of control mAbs for which mapping data were available.

Assays were carried out on four dilutions of NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10. Relative binding of mAb at each concentration to the various transfectants was cumulated (Fig. 7.3) and relevant statistical analysis of the cumulative binding scores is shown in Table 7.3. The most striking effect is abrogation of binding of all four mAbs by the substitution 70D for 70Q (corrected p value, < 0.001). These data are consistent with the previously observed negative binding of all four mAbs to DRβ*0402 and to all other molecules containing DRβ1*:70D (**CHAPTER 6**). In a separate experiment (shown in Table 7.4) the substitution 70Q→R resulted in significant loss of NFLD.D10 binding and total loss of NFLD.D8, NFLD.D3 and NFLD.D2 binding, thus confirming previous findings with 70R positive wild type molecules (Table 7.1 and unpublished data). No other substitutions on the alpha helix of DRβ1*0401 molecules altered NFLD.D10 binding. However, the substitution 57D→S resulted in increased binding of NFLD.D8, NFLD.D2 and NFLD.D3, while the substitutions 67L→I, 71K→R, 71K→E, 74A→E, and 86G→V decreased the binding of one or more of the antibodies (Fig. 7.3).

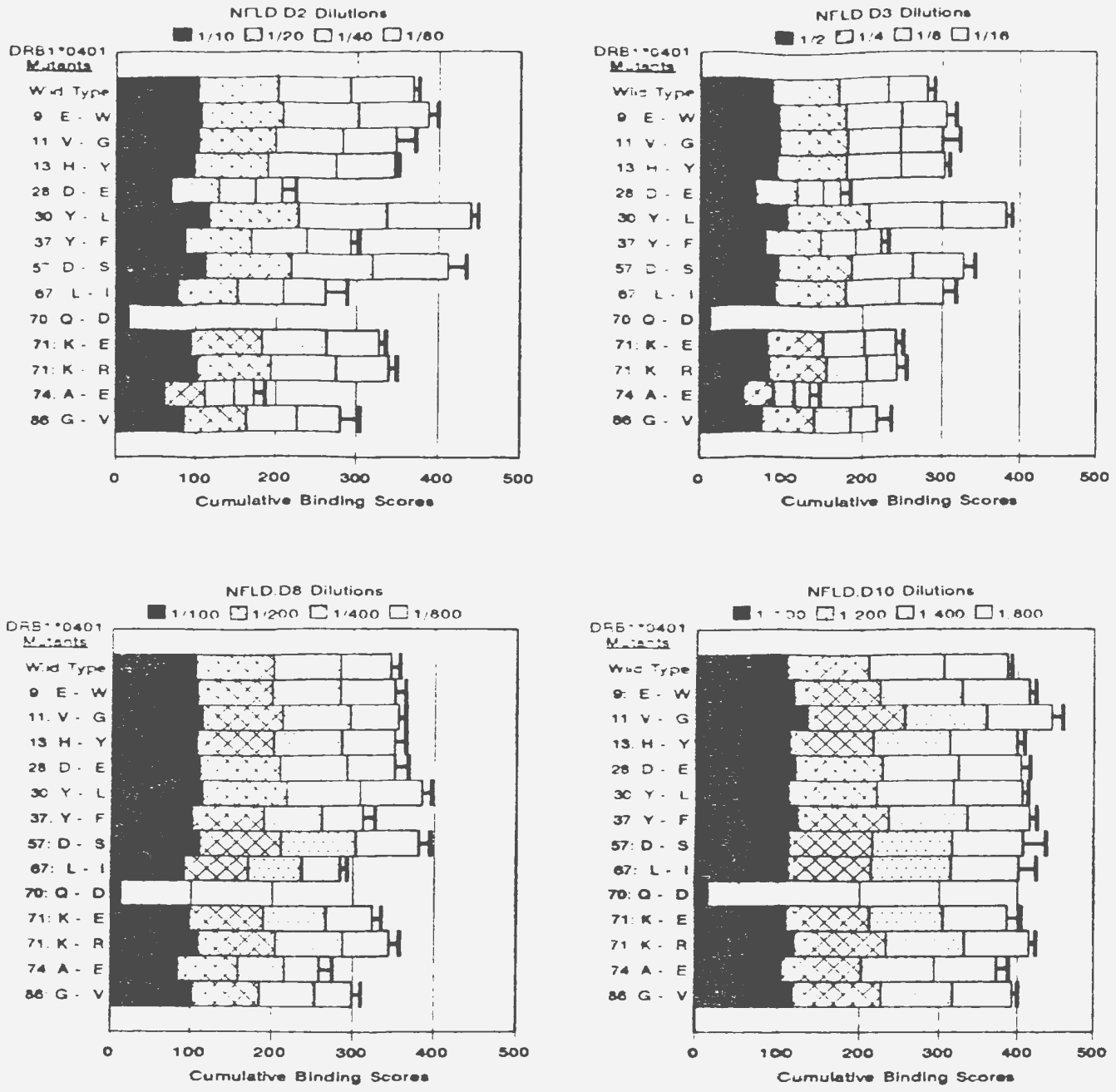

Figure 7.3 Epitope mapping for NFLD.D2, diluted 1/10-1/80; NFLD.D3, diluted 1/2-1/16; NFLD.D8, diluted 1/100-1/800; NFLD.D10, diluted 1/100-1/800 with DRβ1*0401 mutants shown in Fig 7.2. The relative binding of each mAb to each DRB1*0401 mutant is shown on a cumulative scale, with each division in a histogram representing the % binding of each respective dilution of mAb to the various cells.

Significant alterations in mAb binding to mutants were ascertained by Bonferroni corrected p values (Table 7.3); they include NFLD.D2 binding to 67I ($p< 0.01$), 74E ($p<0.001$) and 86V ($p<0.05$); NFLD.D3 binding to 74E ($p< 0.001$); NFLD.D8 binding to 67I ($p<0.05$) and 74E ($p<0.01$). Alterations in binding caused by replacements 57D→S, 71K→R and 71K→E were insignificant (corrected p value, >0.05).

Three substitutions on the floor of the groove (9E→W, 11V→G, 13H→Y) had only trivial effects on binding (Fig. 7.3); for example, increased NFLD.D10 binding to the 11G mutant was insignificant (corrected $p > 0.05$). However, the binding of NFLD.D2, NFLD.D3 and NFLD.D8, but not of NFLD.D10 was modified considerably by at least one of the other substitutions (28D→E, 30Y→L and 37Y→F). The statistical analysis (Table 7.3) shows that reductions in NFLD.D2 and NFLD.D3 binding to the 28E mutant were significant ($p < 0.001$ and < 0.01, respectively), while NFLD.D8 binding was unaffected. Increased NFLD.D3 binding to the 30L mutant was also significant ($p < 0.01$), while increased NFLD.D2 and NFLD.D8 binding to the same mutant and reduced binding by all three mAbs to the 37Y mutant did not reach significance when the Bonferroni correction was applied.

Table 7.3 Monoclonal antibody binding to wildtype and mutated DRβ*0401 molecules, expressed as mean cumulative scores[a] and analyzed by ANOVA[b].

Amino Acid Change	NFLD.D2			NFLD.D3			NFLD.D8			NFLD.D10		
	X[c]	SEM[d]	p[e]	X	SEM	p	X	SEM	p	X	SEM	p
Wild Type	366	6.8		277	10.7		347	10.6		385	1.7	
28: D→E	205	16.8	<0.0001***	170	13.0	0.0002**	352	14.4	0.074	404	7.8	0.37
30: Y→L	440	7.9	0.007	375	8.1	0.0004**	384	11.2	0.038	406	6.9	0.32
37: Y→F	291	9.0	0.0061	218	5.9	0.01	313	13.7	0.057	415	4.7	0.16
67: L→I	253	27.3	0.0004**	296	19.3	0.34	283	5.6	0.0024*	401	22.1	0.45
70: Q→D	12.2	8.8	<0.0001***	8.8	2.5	<0.0001***	8.2	2.2	<0.0001***	10.8	2.8	<0.0001***
74: A→E	171	12.5	<0.0001***	131	9.3	0.0001***	258	15.3	0.002**	372	15.2	0.49
86: G→V	275	25.8	0.002*	214	17.3	0.007	299	11.0	0.012	389	1.7	>0.8

[a] % binding of each mAb sample was cumulated as shown in Fig. 7.3

[b] ANOVA - analysis of variance; [c] X, mean % of replicate samples; [d] SEM, standard error of the mean

[e] p, uncorrected probability values; Bonferroni corrected p values: * = p<0.05; ** = p<0.01; *** = p<0.001

7.3.3 Reconstitution of Antibody Binding Epitopes in non-DRβ1*0401 Context

The above studies showed that 70Q was critical to these antibody binding epitopes, and that other residues contributed to epitope formation. It was, therefore, pertinent to see whether these epitopes could be reconstituted by making a simple 70D→Q replacement in otherwise non-reactive molecules. For example, DRβ1*0402 has the same framework as the prototype DRβ1*0401 with the exception of substitutions 67I, 70D, 71E and 86V, while DRβ1*1101 carries substitutions 67F, 70D and 86V, but differs contextually. The data presented in Table 7.4 show either full or partial reconstitution of all four epitopes on the DRβ1*0402:70D→Q mutants. Similarly, the other epitopes, except for NFLD.D2, were reconstituted on DRβ1*1101:70D→Q.

Since NFLD.D10 was previously shown to bind to certain wild type DR molecules containing DRβ1:70R (Table 7.1, unpublished observations), an attempt was made to recreate this and the other epitopes by substituting 70R for 70Q or 70D. It can be seen from Table 7.4 that DRβ1*1101:70D→R resulted in positive NFLD.D10 binding,whereas DRβ1*0401:70Q→R resulted in a profound loss of binding. Aside from trivial binding of NFLD.D8 to DRβ1*1101:70D→R molecules, none of the other antibodies reacted with these mutants. The absence of these antibody binding epitopes, particularly that of NFLD.D10, on DR3 molecules was curious since DRβ1*03 chains carry 70Q.

Table 7.4. mAb binding[a] to mouse cell transfectants expressing wild type (WT) and mutated DRB1* genes.

NFLD. mAb	0401: WT	0401: 70 Q→R	0402: WT	0402: 70 D→Q	1101: WT	1101: 70 D→Q	1101: 70 D→R	0301: WT	0301: 73 G→A
D10	+++	±	-	+++	-	+++	+++	-	+++
D8	+++	-	-	+++	-	++	±	-	+
D3	+++	-	-	+++	-	++	-	-	±
D2	+++	-	-	++	-	±	-	-	-

[a] mAb binding was measured by immunofluoresence and double normalized as described in methods; -, <10%; ±, 11-25%; +, 26-50%; ++, 51-75%; +++, >75%.

However, it differs from DRβ1*04 chains at amino acid positions 73, 74 and 77 on the alpha helix, and at several positions on the beta strands. Based on the finding that NFLD.D10 binding is unaffected by position 74 (Fig 7.3) and the knowledge that position 73 is important for an antibody binding epitope on DR3 molecules (Nelson et al., 1992), it was predicted that 73G on DR3 molecules might prevent the formation of the NFLD.D10 binding epitope. To test this hypothesis, the antibodies were analyzed on transfectants expressing wild type and mutated DRβ1*0301 molecules in which 73A (DRβ1*0401 context) was substituted for 73G. As predicted and shown in Table 7.4, there was substantial reconstitution (80%) of NFLD.D10 binding; there was also partial reconstitution (45%) of NFLD.D8 binding. However, the binding epitopes for NFLD.D2 and NFLD.D3, which were shown to be the most sensitive to DR context, were not reconstituted.

7.3.4 Peptide Loading of the Groove Influences the Topography of Shared Epitopes on the Alpha Helix

The above data indicated that optimal expression of some of these epitopes required a contribution from residues in the HLA groove that are predicted to bind peptide. This, together with altered binding to DR molecules expressed on mouse cells (Fig.7.1), suggested that peptide in the groove might also be involved. To further

investigate this idea, an antigen processing mutant, T2, was employed; this cell line was previously shown to express transfected DR3 molecules that were conformationally unstable and associated with a large invariant chain peptide (Riberdy and Cresswell, 1992; Sette et al., 1992). Thus, T2 cells, which were transfected with DRA and DRB1*0401 genes (T2-Dw4), were used as targets in CELISA in which several different concentrations of antibody were used.

The expression of DR4 molecules on T2-Dw4, as detected by the DR4-specific mAb (NFLD.D1), was approximately 80% of that found on a normal BCL (WT51) which expresses wild type DRβ1*0401 (data not shown). Binding of NFLD.D2 and NFLD.D8 to the T2-Dw4 mutant was profoundly reduced even when the antibodies were used at saturating concentrations (Fig. 7.4).

NFLD.D3 binding was also conspicuously decreased, requiring 100x more antibody to give 50% binding. NFLD.D10 binding, which was earlier shown to be the least affected by DR context, was the least modified with about a 5-fold reduction in binding to T2-Dw4 as compared to the normal BCL. Thus, the data support the hypothesis of peptide involvement since those same mAbs whose binding patterns were most affected by cell lineage and/or DR context, also had significantly altered binding to T2-Dw4.

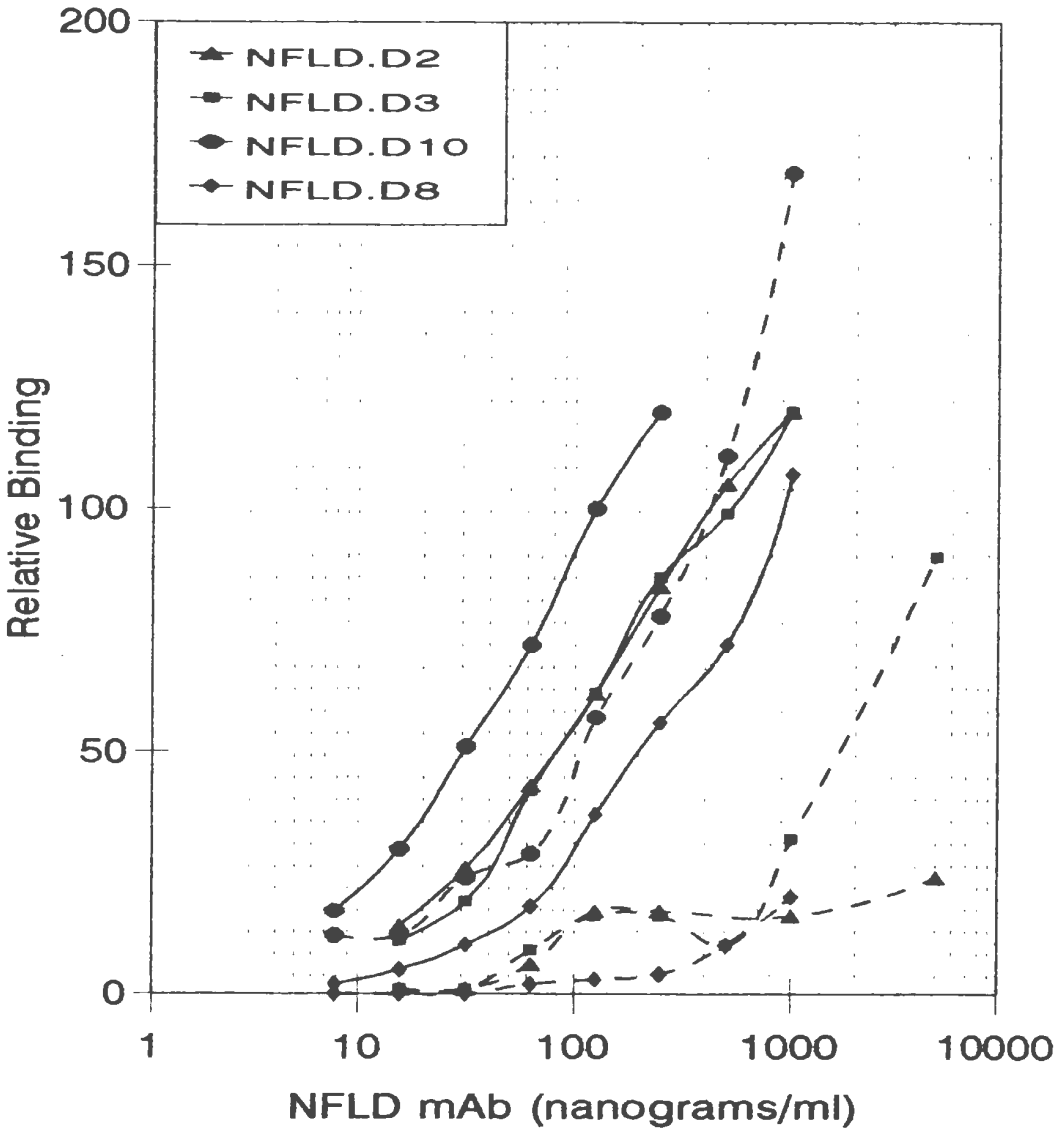

Figure 7.4. Comparison of mAb binding to a homozygous BCL, WT51 (solid line) expressing endogenous DRB1*0401 and mAb binding to an antigen processing mutant, T2-Dw4 (broken line) expressing transfected DRB1*0401. Several concentrations of mAbs (displayed in inset) were tested in CELISA and the results are shown as relative binding, which was calculated as described under methods.

7.4 DISCUSSION

The critical amino acids in the shared sequences (67-86) on those DRβ1 chains that are implicated in RA are oriented so that their side chains are involved in peptide binding and/or T cell recognition (Brown et al., 1993). The importance of positions 67, 70, 71 and 74 was implied from the discovery that DR4 variants which are neutral or confer protection against the development of RA have major substitutions at one or more of these positions. Thus, DRβ1*0402 carries a conservative substitution at position 67 (isoleucine for leucine) and non-conservative substitutions at positions 70 (aspartic acid for glutamine) and 71 (glutamic acid for lysine or arginine), while β1*0403 carries a non-conservative substitution at position 74 (glutamic acid for alanine). In both variants, the non-conservative replacements impart a negative charge on this region (Watanabe et al., 1989). Further support for the importance of this region in immune responsiveness comes from a mouse model in which the equivalent positions 67, 70 and 71 on the I-Aβ chain were shown to be critical in determining susceptibility or resistance to the development of an experimentally-induced autoimmune response in H-2^{b} and H-2^{bm12} mice (Mengle-Gaw et al., 1984; Hochman and Huber, 1984). The concept that a shared structure containing these critical residues is somehow implicated in the development and pathogenesis of RA is tenable, but the mechanism by which it does this has remained an enigma. It seems likely that the framework of the different DR molecules that

carry the RA "susceptibility sequence" and the capacity of these molecules to bind and orient various peptides are important in determining the topography of the shared structure. Thus, a structure formed by a certain sequence on DR4 molecules may have a different conformation from that formed by the same sequence on DR1 molecules. The experimental evidence from this study in which mAbs were used as probes to map the topography of these shared regions, supports this idea.

The epitopes for the four antibodies in this study are formed by residues in a sequence on DR molecules that are implicated in susceptibility to the development of RA and possibly other diseases with an autoimmune component. Subtle variations in these epitopes, which were perceived from differential antibody binding with relevant DR molecules expressed on mouse and human cells, were further clarified by epitope mapping. From these studies, emerged a picture of overlapping structures with 70Q forming the core, but with other residues making a significant contribution to the formation of each epitope (Fig. 7.5). These residues are at positions 86, 74, 67 and 28 for NFLD.D2; positions 74, 28 and 30 for NFLD.D3; positions 74 and 67 for NFLD.D8. Evidence was also provided that 73A is required for at least some of these epitopes. For example, the NFLD.D10 binding epitope, and to a lesser extent that of NFLD.D8, were created on DRβ1*0301 by the substitution 70G→A, thereby, generating the sequence 70-74:QKRAG. Thus, the footprint of the NFLD.D10 epitope may only involve residues 70-73, while that of NFLD.D8 also requires 74A and 67L for optimal expression.

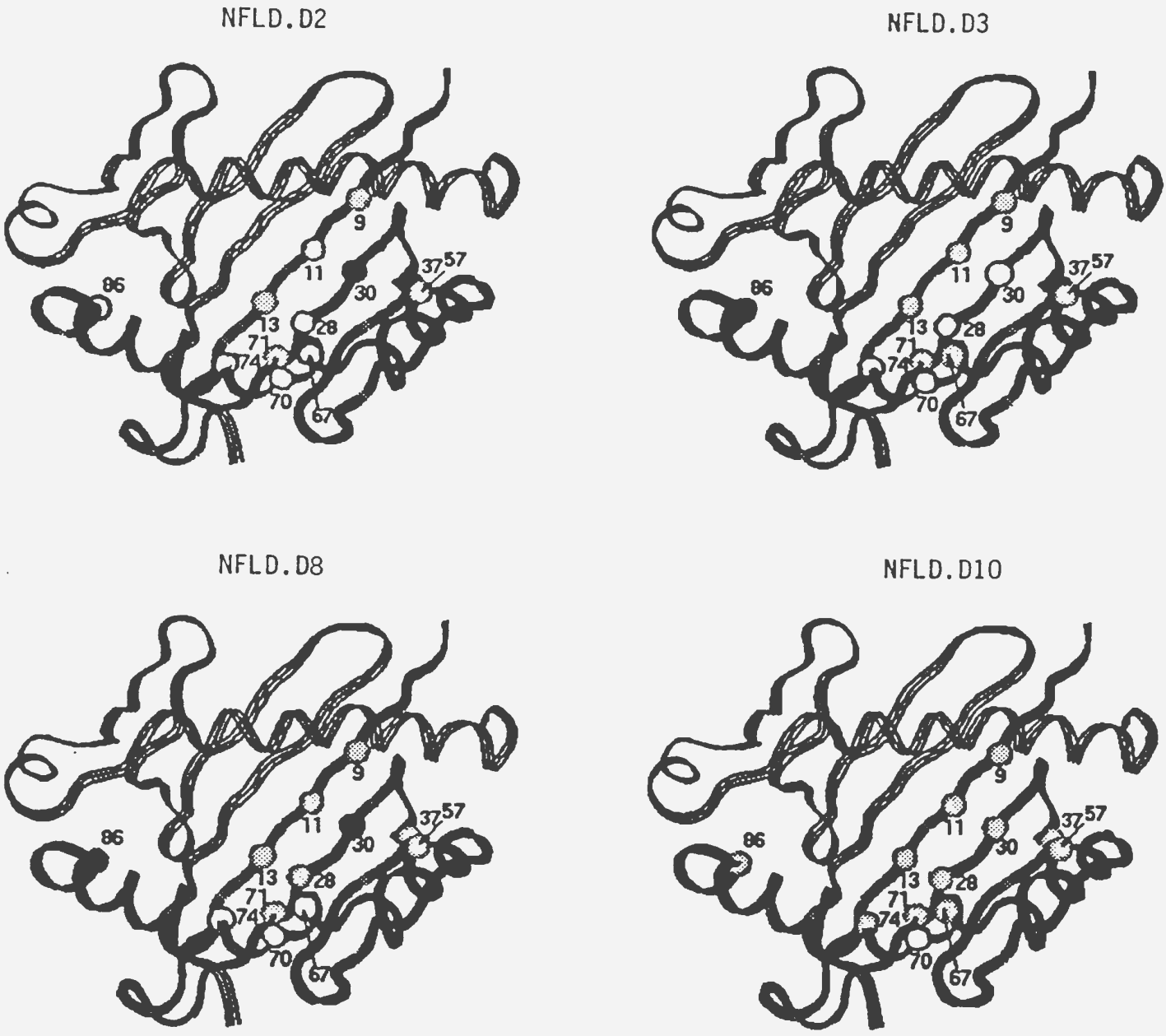

Figure 7.5 Location of amino acid residues in DRβ1*0401 molecules that contributed to antibody binding epitopes. Alterations are indicated by: open circles, amino acid substitutions that caused a significant alteration to the epitope; closed circles, an insignificant modification to the epitope; shaded circles, no effect on the epitope.

The epitopes for NFLD.D2 and NFLD.D3 appear to be further influenced by more remote parts of the alpha helix and spread onto a region on the peptide binding site that is normally occupied by peptide. The clearest evidence for peptide involvement in these epitopes was the profoundly altered antibody binding to DRβ1*0401 molecules when they were expressed by the antigen-processing mutant, T2. This cell line expresses conformationally unstable class II molecules due to defective loading of the MHC groove (Riberdy and Cresswell, 1992; Sette et al., 1992). As might be expected, NFLD.D10 was the least affected by the absence of normal peptide insertion into the groove, while the binding of NFLD.D2, NFLD.D3 and NFLD.D8 were markedly diminished.

Interpretation of these data and delineation of these epitopes were aided immensely by the recent publication of the 3-D structure of a DR1 molecule (Brown et al., 1993). Amino acid positions 70 and 73 are critical residues for NFLD.D10 which is essentially unmodified by either DR context or by peptide in the groove. In the DR1 crystal structure the side chains of position 70 have ambigous electron density (Brown et al., 1993); these data suggest that the side chains of this position, like those of position 73, point up and away from the peptide binding site; thus, they are able to make direct contact with either antibody or T cell receptors. In contrast, are the composition of shared epitopes, such as NFLD.D2 and NFLD.D3, which vary considerably with DR context and peptide occupancy. For example, two substitutions 28E and 74E which significantly altered the structure of these epitopes are within a

polymorphic cluster (β 13, 26, 28, 71, 74, 78) which is important for peptide binding and orientation (Brown et al., 1992; Racioppi et al.,1993; Coppin et al., 1993). Position 86, which is at the entrance to the groove and appears to be involved in peptide anchoring (Demotz et al., 1993), also contributed to the NFLD.D2 binding epitope. Thus, the locations of the amino acids that shape these epitopes are consistent with the idea that peptide occupancy of different HLA grooves can induce epitope variation; whether or not peptide directly contributes to some epitopes or acts indirectly by conformational induced alterations cannot be deduced from these studies.

Other studies have identified shared structures, that form antibody or alloreactive T cell epitopes on the alpha helix of DR molecules, that correlate with the presence of an RA "susceptibility sequence" (Winchester and Gregersen, 1988; Duquesnoy et al., 1984; Hiraiwa et al., 1990). One of the first, designated MC1, was defined by Duquesnoy et al, (1984) using alloantibodies whose binding correlated significantly with the presence of DR4 and DR1; however, MC1 was occasionally found on non-DR4 molecules, a finding that is similar to these antibody epitopes. Particularly relevant to this work is a study done by Hiraiwa et al (1990), in which another mAb with MC1-like specificity, CCCL20, was mapped to residues 70Q, 71R and 67L on DRβ1*0404 molecules. Its epitope is similar to those of NFLD.D2 and NFLD.D8, since all three are affected by positions 67 and 70, whose side chains face solvent, but it differs at positions 71 and 86. Position 71 had little impact on

NFLD.D2 or NFLD.D8, while the position 86 had no effect on CCCL20. Whether or not residues on the floor of the groove contribute to CCCL20 was not tested, but the fact that the authors were able to reconstitute this epitope on a DRB1*1101:70D→Q mutant, suggests that DR context may not be that important. The failure of NFLD.D2 to be reconstituted by similar mutations in DRB1*1101, and its overall dependence on DR context and peptide binding for optimal expression, suggest that its binding epitope more closely resembles that of an alloreactive T cell epitope.

The idea that a consensus sequence on the alpha helix of some HLA-DR molecules (QKRAA/QRRAA in DR4 and DR1 or RRRAA in DR10) generates a conformationally equivalent determinant, that predisposes to RA, was partly based on the identification of another serological epitope (109d6) found on the cells of most RA patients (Winchester and Gregersen, 1988; Merryman et al., 1988). Subsequently, it was shown that the high correlation of this epitope with RA was due to its presence on DR53 molecules, whose gene is in linkage disequilibrium with DR4, but which is not associated with RA. However, 109d6 positive molecules also include DR10 which is associated with RA (Nelson et al., 1992; Ollier and Thompson, 1992; Sanchez et al., 1990) and DR9 which was recently shown to be associated with RA in Chile (González et al., 1992). Interestingly, 109d6 positive molecules contain 70-73:RRRA, which was shown in this study to form a cross reactive epitope for NFLD.D10. Surprisingly, the NFLD.D10 epitope was recreated

on DRβ1*1101 carrying the substitution 70Q→R, but not on DRβ1*0401 by the same substitution. Thus, it seems that the consensus sequence QKRAA/ QRRAA/ RRRAA has the potential to generate a similar configuration but which may be dependent on DR context and peptide in the groove.

The finding that these four antibodies bind to cells that express certain HLA-DR2 (DR15 or/and DR51) is intriguing since DR2 is not associated with RA (Nelson et al., 1992; Ollier and Thompson, 1992). Examination of the amino-acid sequence data however shows that a slight modification of the key sequence QKRAA or QRRAA is found on some DRβ chains from DR2 haplotypes. A possible key to understanding this apparent paradox of epitope presence but little or no RA association, is that each DR2 cell expresses both DRβ1* and DRβ5* chains. The fact that QARAA seems not to predispose to RA may be because the closely linked gene in each case encodes a similar "protective" sequence, the same as that of DRB1*0402. Thus the potentially susceptible DRβ1*15 molecules may be counterbalanced by 70D in the sequence (70-74:DRRAA) on DRβ5*01; conversely, the potentially susceptible DRβ5*02 on DR16 positive cells may be counterbalanced by the protective 70D in DRβ1*16 molecules. Alternatively, the fact that DRβ1*15 and DRβ5*02 chains carry 67I and 71A, while the RA-associated molecules carry 67L and 71K/R may also explain the lack of DR2 association with RA.

It is tantalizing to note that the floors of DR15 molecules are more like those of DR4 molecules than are the other non-DR4 molecules (DR1, DR14 and DR10)

that are implicated in RA. Furthermore, both DR15 and DR4 are associated with several rheumatic diseases that have an autoimmune component. For example, a shared sequence consisting of residues 26-33:FLDRYFY on the beta strands, together with 70Q and 73A, on both DR15 and DR4 β chains have been predicted to form a "shared epitope" that is associated with the production of U1-70kd autoantibodies in mixed connective tissue disease (Kaneoka et al., 1992). In addition, the development of chronic arthritis in Lyme disease is associated with DR2 and the same DR4 alleles that predispose to RA (Steere et al., 1990). Recently, Goronzy et al (Weyand et al., 1992) also showed giant cell arteritis is highly associated with DRβ1*0401 and 0404/8 as well as with other DRβ chains that carry a shared sequence on the floor of the groove.

The work presented in this study provides direct experimental evidence that the topography of shared structures, which map to the β chain alpha helix of the peptide binding site is influenced by spatially-related residues that are located on the floor of the groove. The information gained about the NFLD.D2 binding epitope, which most closely resembles the putative RA"shared epitope", suggests that the conformation of the RA "susceptibility epitope" will also vary on the implicated DR molecules. For example, both epitopes share the pivotal glutamine at position 70 and require a contribution from leucine at 67, alanine at 74 and glycine at 86; however, they may differ regarding the involvement of position 71, at which a basic amino acid is considered important for the RA "susceptibility epitope." That the substitution of

glutamic acid (found in DR1, DR10 and DR14) for aspartic acid (DR4) at position 28 significantly altered the NFLD.D2 epitope, both in natural variants (DR1 & DR14) and the DRβ1*0401 mutant, suggests that this position contributes to the RA susceptibility epitope. Because β28 is spatially proximate to β70-74:QK/RRAA, and because of its importance, along with β71 and β74 in a peptide-binding pocket (Racioppi et al., 1991; Coppin et al., 1993), a substitution at this position could have serious implications for the binding and/or orientation of an arthritogenic peptide(s) by these molecules; we envisage that either the substitution, itself, and/or peptide binding could conformationally-alter this epitope. The fact, that non-DR4 RA-associated molecules (28E positive) do not carry the same risk for susceptibility and disease severity in RA, supports this idea. Furthermore, the critical dependence of the NFLD.D2 epitope on peptide in the groove reinforces the straightforward idea that the RA susceptibility sequence may cause its effects by determining T cell reactions. Whether this is achieved by affecting the T cell repertoire through an influence on T cell selection in the thymus, or by a peripheral mechanism, is not yet clear.

CHAPTER 8

DIFFERENTIATION OF THE T-CELL DEFINED DR4Dw4 SPECIFICITY FROM OTHER DR4 SPECIFICITIES WITH MONOCLONAL ANTIBODIES[1]

8.0 ABSTRACT

The T-cell defined DR4 subtypes, Dw4 and Dw14, have not previously been discernable by antibodies. It is expected that this is due to the conservative nature of the substitutions (lysine to arginine at position 71 and glycine to valine at position 86) and their involvement in peptide binding. Here we describe two mAbs, NFLD.D11 and NFLD.D12, having the precise specificities of alloreactive T cell clones in discriminating Dw4 from other DR4 and non-DR4 subtypes. NFLD.D11 binds only to Dw4-positive cells while NFLD.D12 cross-reacts with 25% of Dw14-positive B cell lines. The data suggest that these antibodies are peptide-dependent as they exhibit both tissue and species specificity and do not bind to Dw4 molecules that are expressed on antigen processing mutants such as T2. Furthermore, binding of NFLD.D11, but not of NFLD.D12 could be partially reconstituted on mouse cell transfectants if they were first pulsed with a human cell extract. Although these antibodies have similar attributes to alloreactive T cell clones, it has not yet been resolved if their epitopes are the same.

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[1] **Manuscript in preparation**

8.1 INTRODUCTION

Nucleotide sequencing has revealed that the serologically-defined HLA-DR4 specificity comprises several DRB1 allelic variants (Stastny et al., 1992; Bodmer et al., 1992); eight of these have previously been defined by T-cell recognition methods. From analysis of the inferred amino acid sequence data (Barbetti et al., 1992; Marsh et al., 1992) and epitope mapping studies with anti-DR4 monoclonal antibodies (Alber et al, 1989; Maurer and Gorski, 1991; **CHAPTER 6**), it appears that structures which determine common serological epitopes depend on a combination of amino acid sequences in the first and second hypervariable regions of the β_1 domain and/or limited polymorphism in the β_2 domain.

The T-cell defined subtypic determinants on DR4 molecules map to the alpha helix of the β chains and vary from each other by only one to four amino acids. For example, Dw4 differs from Dw14 by only two conservative amino acids, lysine (K) for arginine (R) at position 71 and glycine (G) for valine (V) at position 86. These positions are located on the alpha helix in such a way that their side chains are involved in peptide binding (Brown et al., 1993). Since these residues are also found on certain non-DR4 β chains such as those of DR1 and DR14, to which a Dw4- or Dw14-specific T cell clone does not bind, it has been predicted that these determinants depend on critical residues on the alpha helix, the DR framework and probably peptides in the MHC grooves (Lechler et al., 1991; Benoist and Mathis,

1991; Obato et al., 1992; Sherman and Chattopadhyay, 1993).

In view of these findings, it is perhaps not surprising that despite extensive searches during several HLA workshops, no reliable allo-antisera were identified that correlated precisely with the T-cell defined subtypes. However, serological splits of DR4 were proposed based on weak reactivity patterns that correlated with DR4 β chains containing either alanine (Dw4, Dw14, Dw15) or glutamic acid (Dw13 and DwKT) at position 74 (Williamson et al., 1984, 1992). It is of interest that position 74 is also involved in peptide binding.

The data presented in this paper demonstrate that two subtypic differences (Dw4 and Dw14) of DR4, previously only amenable to T-cell typing, were clearly identifiable by two monoclonal antibodies. Furthermore, evidence is presented that their binding is remarkably similar to that of T cells, since peptide in the MHC groove is required for formation and integrity of the epitopes.

8.2 METHODS

8.2.1 Cells

Human B cell lines were obtained mainly through the 9th and 10th International Histocompatibility Workshop (IHW) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, penicillin

and streptomycin (all from Flow Laboratories, McLean, VA). The B cell line, SAVC (10th IHW number, 9034), which is homozygous for DR4Dw4, DQ8, DP10, was used for immunization of mice. Peripheral blood B cells (B-PBL) were prepared from normal blood donors in a two-step procedure; Lymphoprep (Cedarlane Laboratories, Hornby, ONT) was first used to isolate the mononuclear cells, which were then depleted of T cells using sheep red blood cells (Qualicum Scientific Limited, Nepean, ONT) that had been treated with neuraminidase (Sigma, St. Louis, MO) as described previously (Boyum 1966; Weiner et al., 1973). Different types of transfectants were also used for some of these studies. Mouse L cell transfectants (DAP-3 DR4 and L243.8) which express Dw4 have been described previously (Inoko et al., 1992; Chapter 6). The mutant B-cell lines, T2, an antigen processing mutant (Riberty and Cresswell, 1992) and BLS-1, an EBV-transformed B-cell line derived from a patient with Bare Lymphocyte Syndrome (Hume et al., 1989), were transfected with DRA and DRB*0401 genes; these two mutant cell lines were kindly donated by Dr. G. Nepom.

8.2.2 Monoclonal Antibodies

Two monoclonal antibodies NFLD.D11 and NFLD.D12 (IgM kappa isotypes) were derived from a Balb/c mouse that had been primed with affinity purified HLA molecules, which were isolated from a cell lysate of SAVC using immunometallic

beads (Dynal Inc, Great Neck, NY). The complete methodology for the preparation of HLA-loaded beads has been described by Qi, 1993. Essentially a mAb (NFLD.M67) which detects a monomorphic determinant on HLA-DP molecules (Marshall et al., 1992) was coated on Dynal beads bearing goat anti-mouse IgG. These beads were then mixed with the cell lysate and the HLA-loaded beads were removed with a magnet. The beads were injected subcutaneously in complete Freunds adjuvant (CFA) in four sites of a Balb/c mouse and left for 3 months. Three days prior to splenectomy and fusion the mouse was boosted intravenously with 1 x 10^7 SAVC cells. Fusion was carried out using SP2/0-Ag14 cells as previously described (Drover, 1986). Control mAbs included NFLD.D1, which binds to an epitope on the β_2 domain of all DR4 molecules (**CHAPTER 6**), L243, which binds a monomorphic epitope on all DR molecules (Lampson and Levy, 1980), and isotype-matched controls: IgM, D161-X711; IgG1, K040-L631; and IgG2a, A161-L641, which were obtained from Southern Biotechnology Associates, Inc, Birmingham, AL.

8.2.3 Binding Assays

A cellular enzyme-linked immunosorbent assay (CELISA) was used for screening hybridoma supernatants and for specificity analysis as previously described (Drover and Marshall, 1986). Indirect immunofluorescence with flow cytometry was used for some of the analyses using a standard method. Briefly, cells were washed in

phosphate buffered saline containing 0.1% FBS and 0.01% sodium azide; approximately, 2 x 10^5 cells were reacted with 25μl primary antibody or irrelevant Ig, washed and stained with either goat anti-mouse (GAM)-IgG or GAM-IgM, which were labelled with fluorescein isothiocyanate (FITC) or phycoerythin (PE). In some assays double staining was carried out using FITC-CD19 (Jackson Immunoresearch Laboratories) to stain the B cells. Log fluorescence was read using a Becton Dickinson FACS Star Plus.

8.3 RESULTS

8.3.1 Specificity Analysis of NFLD.D11 and NFLD.D12

8.3.1A B Cell lines as targets

Two antibodies, obtained from the same mouse, were selected in preliminary screening as they were reactive with the immunogen (SAVC) but were negative with several other B cell lines. Comparison of HLA antigens expressed on SAVC to those expressed on the negative BCL suggested that both mAbs recognized the DR4Dw4 subtype. After cloning the hybridomas, the mAbs were further analyzed in CELISA on a large panel of non-DR4 and DR4 positive BCL, which included the majority of those shown in **Table 6.1, CHAPTER 6.** Aside from one or two minor reactions (less than 20% binding), they were completely non-reactive with all non-DR4 expressing

BCL (data not shown).

The reactivity pattern for the DR4, DR1 and DR14 positive cells are shown in Figure 8.1. NFLD.D11 bound strongly to all six BCL that express the Dw4 subtype (DRB1*0401) of DR4. NFLD.D12 gave an almost identical reactivity pattern to NFLD.D11, but it also reacted significantly with one of the four lines expressing the Dw14 (DRB1*0404) subtype of DR4.

Minor reactions were also noted with the remaining two Dw14 positive cells as well as with a Dw10 positive cell. Neither NFLD.D11 nor NFLD.D12 bound to cells expressing DRB1*0101, 0102, and 1402, which have previously been shown to encode epitopes shared with DRβ1* 0401, 0404, and 0405 chains (**CHAPTER 7**). These data strongly suggested that NFLD.D11 could differentiate Dw4 from all other DR4 specificities, whereas NFLD.D12 cross reacted with some of the Dw14 positive cells. These specificities were subsequently confirmed by complement-dependent cytotoxicity testing on a panel of 133 BCL including the following DR4 positive variants: nine Dw4 (B1*0401) lines, five Dw10 (B1*0402) lines, four Dw13 (B1*0403/7) lines, seven Dw14 (B1*0404) lines, five Dw15 (B1*0405) lines, and three DwKT2 (B1*0406) lines by S. Marsh at the ICRF in London (personal communication).

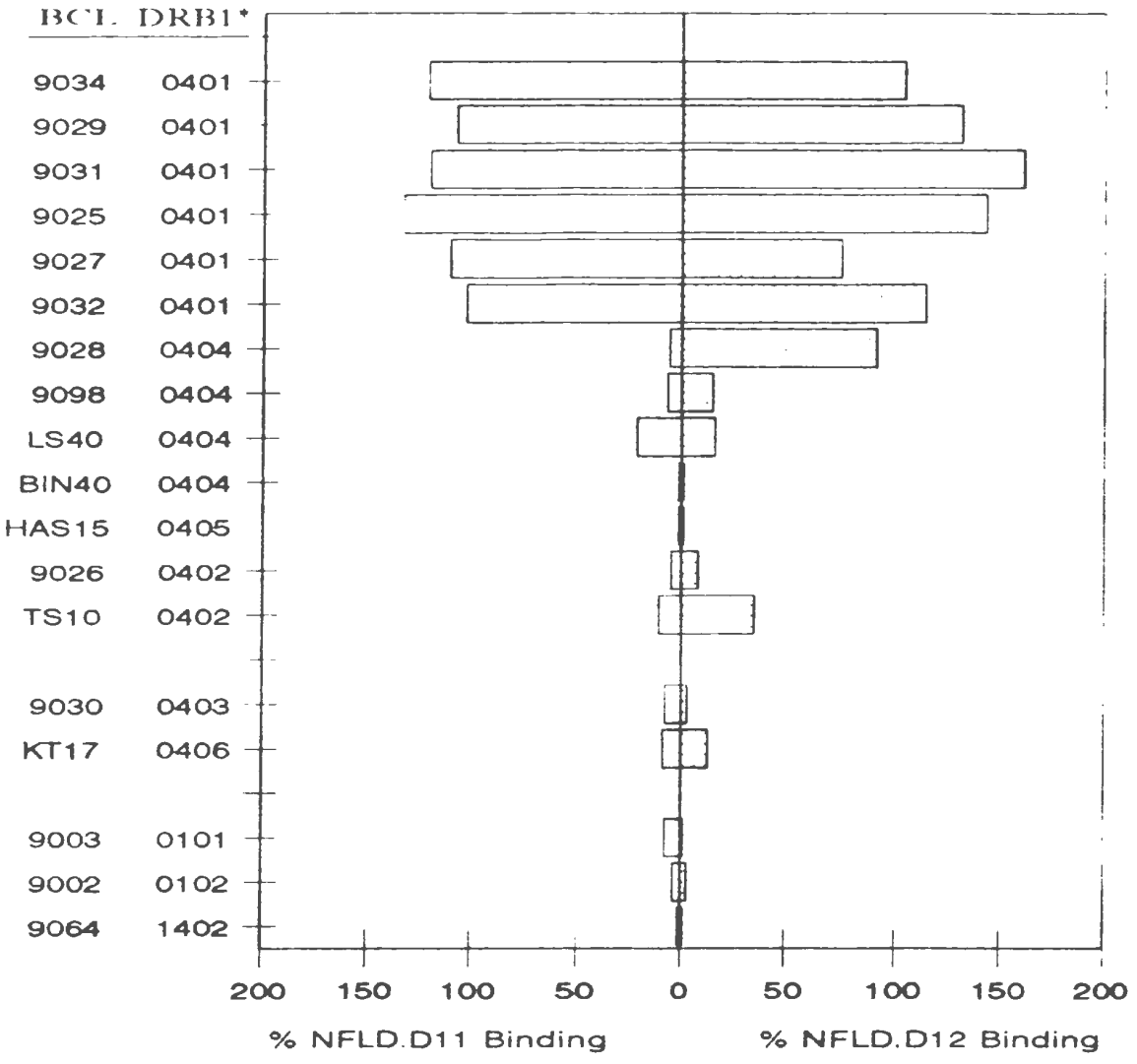

Figure 8.1 NFLD.D11 (left panel) and NFLD.D12 (right panel) binding to BCL expressing either DR4, DR1 or DR14 alleles. Binding was measured in CELISA and the % binding was determined by:

$$\frac{\text{[Optical density (OD) of test] - [OD of background]}}{\text{[OD of L243 positive control] - [OD of background]}} \times 100\%$$

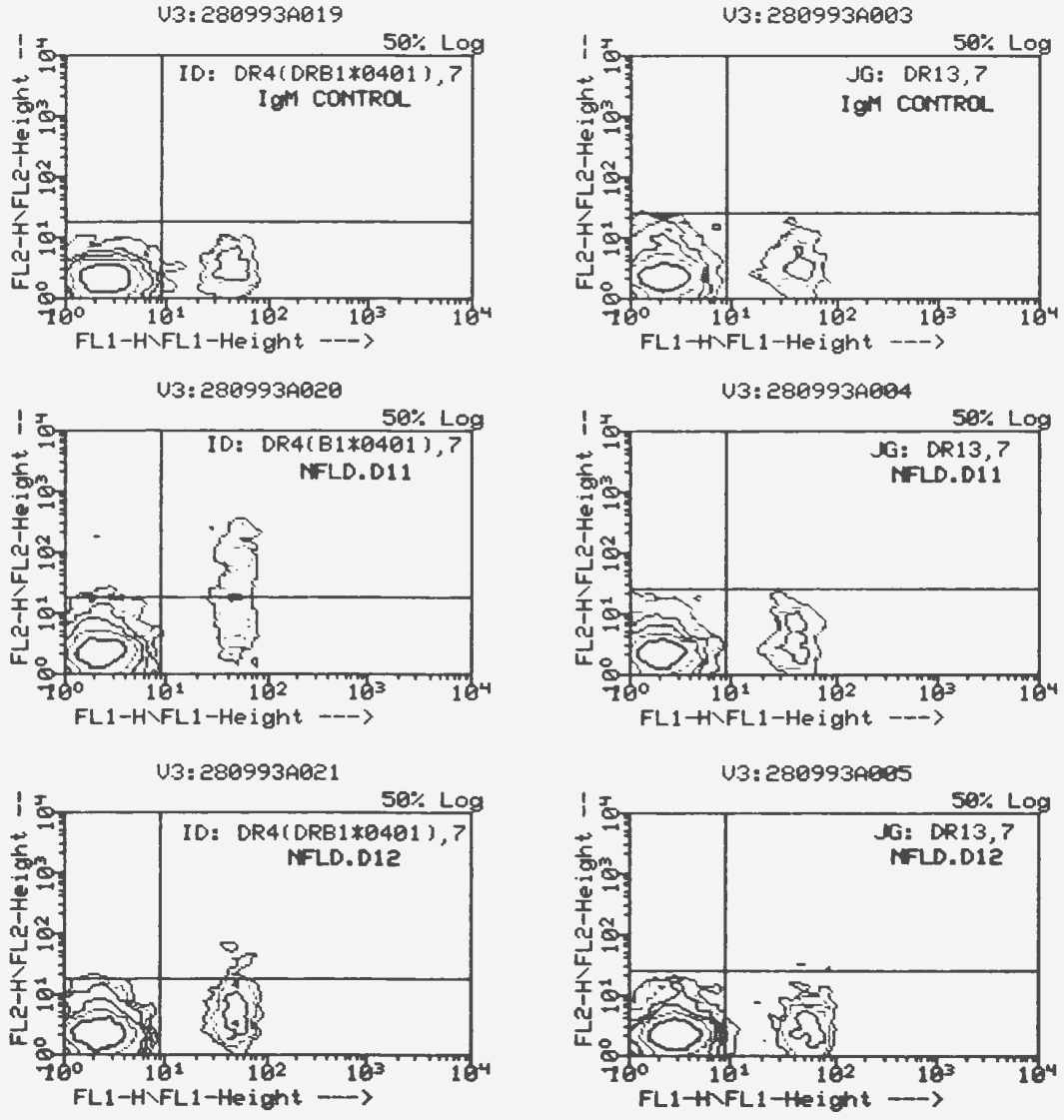

Figure 8.2 FACS analysis of double-stained peripheral blood B cells. Cells were stained with FITC-labelled anti-CD-19 (X axis) and anti-Dw4 mAbs (NFLD.D11 and NFLD.D12) or irrelevant mouse IgM, which were detected with PE-labelled GAM-IgM (Y axis). The panels on the left show Dw4-positive cells [ID: DR4 (DRB1*0401), 7]; the panel on the right show Dw4-negative cells [JG:DR13,7].

8.3.1B Peripheral blood B cells and mouse cell transfectants as targets

NFLD.D11 and NFLD.D12 were also analyzed by flow cytometry on a panel of 34 peripheral blood B cells (B-PBL). The pattern shown for NFLD.D11 and NFLD.D12 binding in Figure 8.2 is representative of the data for all Dw4-positive and Dw4-negative B-PBL. Neither mAb reacted with any of the 14 non-DR4 positive cells, while NFLD.D11 reacted with all 12 Dw4-positive cells, but not with the 8 samples that expressed the DR4 subtypes, Dw13, Dw14 and DwKT2; no cell with Dw10 was available for testing.

NFLD.D11 binding to Dw4-positive cells was between 21% to 60% of the DR-specific positive control (L243) or the B-cell marker CD19. However, NFLD.D12 binding was usually less than 5% of the positive control.

Flow cytometry profiles of NFLD.D11 binding to normal B-PBL and B cells from a chronic lymphatic leukaemic patient (B-CLL) revealed a heterogenous pattern compared to the relatively homogeneous and strong reactivity observed with SAVC (Figure 8.3). It also showed that NFLD.D12 binds slightly better to B-CLL than to the normal B-PBL, but its reactivity is very weak and heterogeneous compared to that with SAVC.

The mAbs were also analyzed on mouse cell transfectants (Figure 8.3). It can be seen that the Dw4-positive transfectant (Dap-3 DR4) strongly bound the DR4-specific mAb (NFLD.D1), but was completely negative with NFLD.D11 and NFLD.D12.

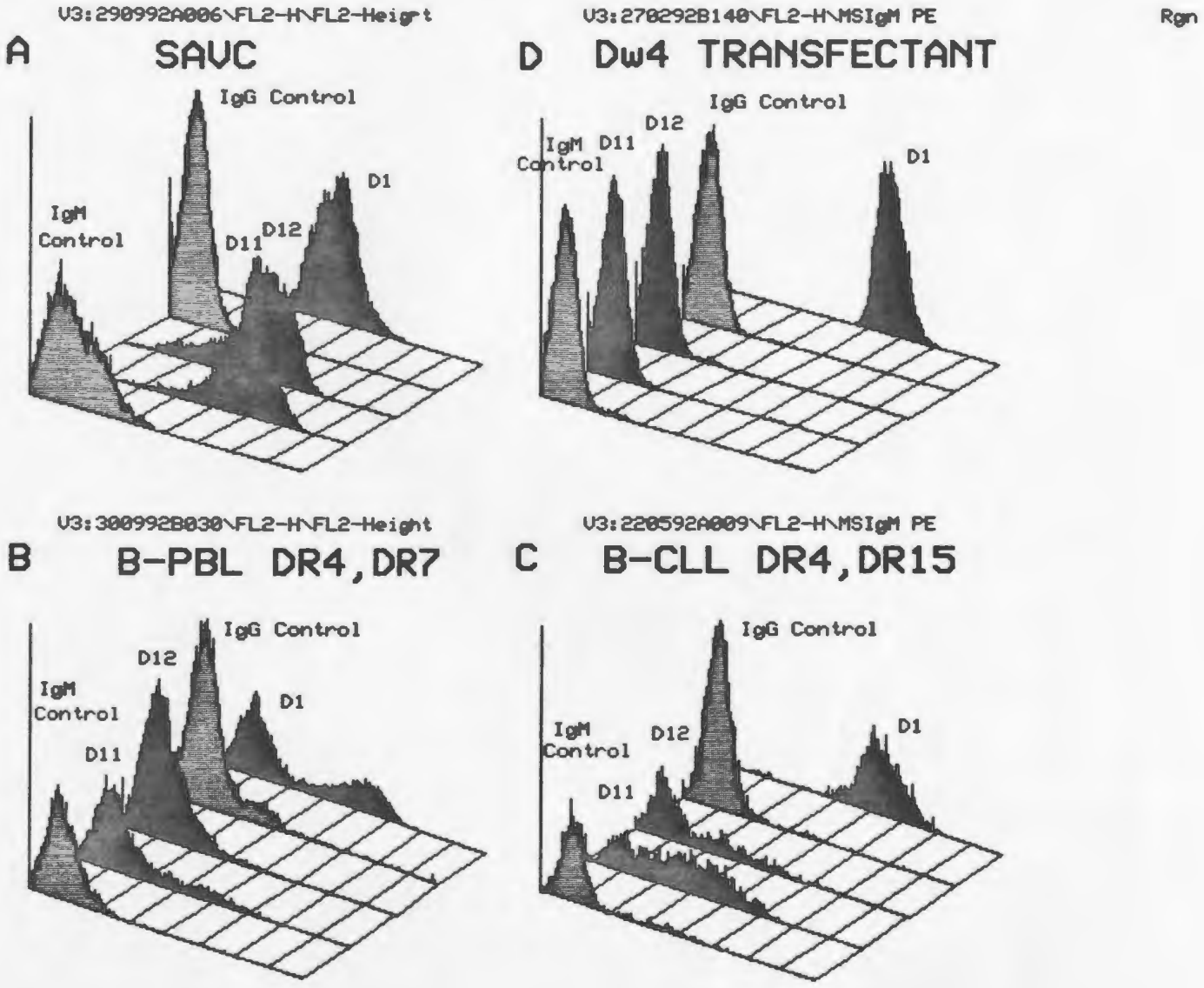

Figure 8.3 mAb binding to Dw4 molecules expressed on different cell lineages. Binding to (A) SAVC, a BCL; (B) B-PBL; (C) B-CLL; (D) Dw4-positive mouse cell transfectant was detected using PE-labelled GAM-IgG + IgM.,

The same results were obtained for L243.4, which is another Dw4-expressing transfectant (data not shown). This failure of both mAbs to bind transfectants and of NFLD.D12 to bind B-PBL is intriguing since the Dw4 genes in both cases are the same. Possible causes for these epitope differences are that these cell types include the presence of different peptides in the peptide binding grooves, differing assembly of the molecules in each case or differing glycosylation.

8.3.2 Contribution of Peptide to NFLD.D11 and NFLD.D12 Binding Epitopes

T-cell discrimination of the Dw4 and Dw14 subtypes is believed to be due to recognition of determinants in which the critical amino acids are peptide-binding residues at positions 71 and 86. It was therefore hypothesized that the negative NFLD.D11 and NFLD.D12 binding to mouse cells expressing Dw4 molecules might be due to differences between mouse and human peptide bound to the Dw4 grooves. To test this idea experiments were done to see if NFLD.D11 and NFLD.D12 binding to Dw4-expressing transfectants could be reconstituted by pulsing them with a crude lysate from either Dw4 positive cells (SAVC) or DR4 negative cells (KAS116, DR1 and PLH, DR7). These cells were disrupted by freeze-thawing, centrifuged and the supernatant collected.

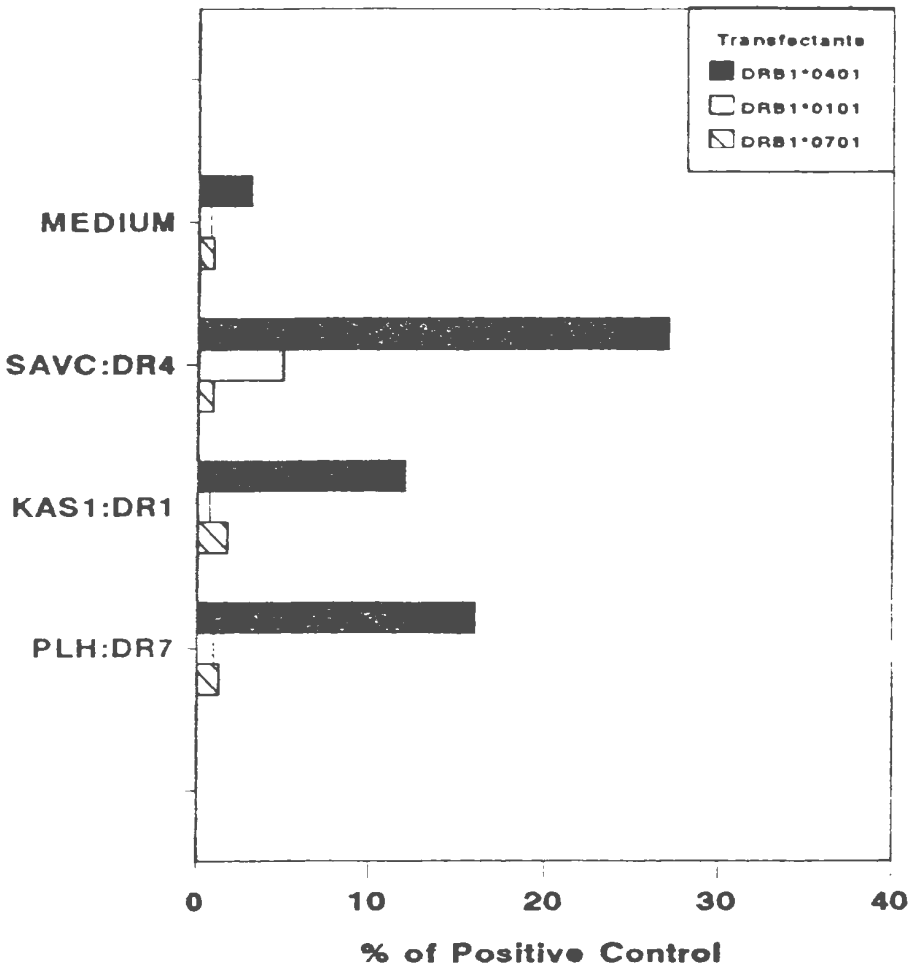

Figure 8.4 Reconstitution of NFLD.D11 binding epitope on mouse cell transfectants. Three different mouse cell transfectants, expressing DRB1*0401 (Dw4), 0101 (Dw1) and 0701 (Dw7) were tested for binding NFLD.D11 after exposure either to culture medium, or to lysates from homozygous BCL: SAVC, DR4Dw4; KAS116, DR1Dw1; PLH, DR7Dw7. The NFLD.D11 binding epitope was partially restored on the DRB1*0401-expressing transfectant with each of the lysates, but the other non-Dw4 transfectants were not affected by exposure to these lysates.

Supernatants, containing crude membranes or the plain culture medium, were used to pulse Dw4-positive and Dw4-negative transfectants for 18 hr, after which time they were tested for their capacity to bind NFLD.D11 and NFLD.D12. Data from one experiment, presented in Fig. 8.4, show that binding of NFLD.D11 was partially restored by this procedure. This phenomenon was not observed for another transfectant (L243.4) which expresses the same DRB gene (data not shown). This is difficult to interpret since both transfectants are derived from the same L-cell fibroblast line (DAP.3) and bind DR4 mAbs similarly; however, morphologically, the two cells appear quite different.

This experiment was repeated sometime later with a different batch of cell lysate; although there was a definite increase in binding to DAP.3 DR4 (β1*0401) pulsed with the lysate, reconstitution was not as marked as in the first experiment. It is notable that NFLD.D12 binding was not reconstituted in either experiment.

8.3.3 DR4Dw4-specific mAbs do not Bind DR4Dw4 Molecules Expressed on Mutant B Cell lines

To further explore the idea that these antibody-binding epitopes are dependent on MHC-peptide complexes, binding studies (FACS and CELISA) were performed using two mutant human B cell transfectants expressing Dw4, namely, T2-Dw4 and BLS-Dw4 (Figure 8.5). The T2 mutant has been shown previously to be

defective in peptide loading of HLA molecules and to express conformationally-altered class II molecules (Sette et al., 1992). It was therefore hypothesized that if these mAbs were peptide-dependent, they would not bind to T2-Dw4 but that they might bind to BLS-Dw4 transfectant. This latter cell line does not express its endogenous class II genes due to a defect in the transcription of its structural genes (Glimcher and Kara, 1992).

Data from a representative experiment using CELISA to measure binding is shown in Figure 8.5. As predicted, neither NFLD.D11 nor NFLD.D12 reacted with T2-Dw4, while both reacted strongly with the normal Dw4-expressing BCL (WT51). Surprisingly neither mAb reacted with BLS-Dw4.

Since the relative binding of the DR4-specific mAb (NFLD.D1) to both these mutants was greater than to WT51, negative binding to the mutants cannot be attributed to inadequate expression of Dw4 molecules. The negative binding of NFLD.D11 and NFLD.D12 to BLS-Dw4 transfectants was at that time puzzling since it suggested that Dw4 molecules expressed on the BLS transfectants might also be defective in peptide loading. This has subsequently been shown to be the case as BLS transfectants are unable to stimulate antigen-specific T-cell clones using intact antigen, but like T2, they can stimulate effectively when exposed to the appropriate peptide (S Kovats, personal communication). Thus, these data further support the concept that Dw4-specific antibodies recognize structures that are similar to the determinants that are recognized by alloreactive T-cells.

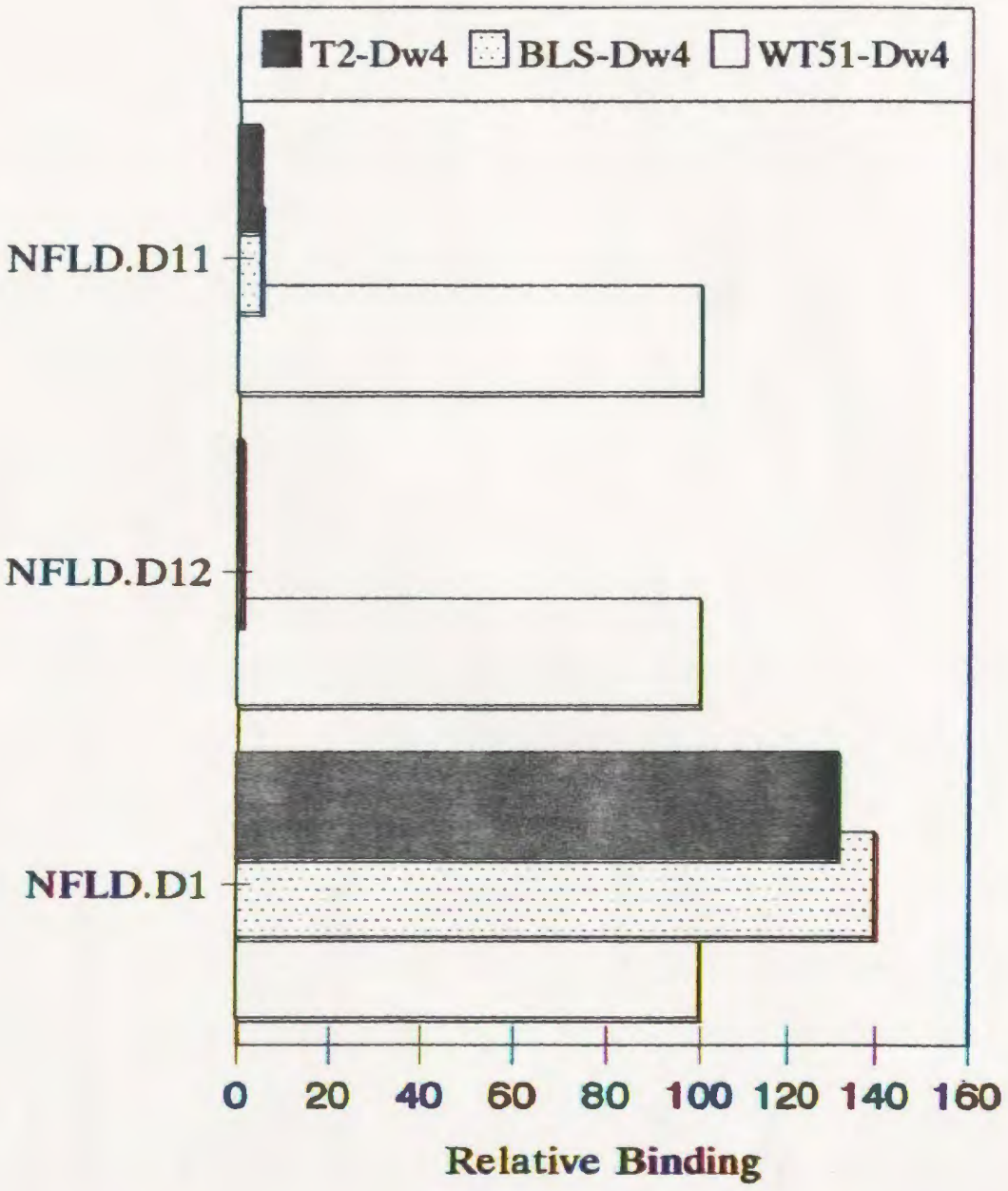

Figure 8.5 mAb binding to Dw4-positive BCL mutants (T2-Dw4 and BLS-Dw4) and on a normal BCL (WT51). Binding was measured in CELISA and is expressed as a percent of the DR positive control, L243, as described for Figure 8.1.

8.4 DISCUSSION

The data presented in this paper clearly demonstrate for the first time, that antibody can differentiate Dw4 from all other DR4 subtypes, including the highly related Dw14 subtype, with the same exquisite specificity as an alloreactive T cell. The rareness of this finding can be attributed to the conservative nature and location of the two amino acids that distinguish Dw4 from Dw14. For example, the key polymorphic difference between the two subtypes is an arginine substitution for lysine at position 71 in both dimorphic variants of Dw14 (B1*0404 and 0408); the latter variant of Dw14 (B1*0408) is more like Dw4 in that it also has glycine instead of valine at position 86. Furthermore, there is substantial evidence implicating both positions 71 and 86 in peptide binding pockets (Coppin et al., 1993; Demotz et al., 1993; Brown et al., 1993). Thus, not only are the critical amino acids conservative and equally charged, but their side chains are buried in peptide binding pockets so that they are inaccessible for TCR or antibody binding.

What then is the nature of the allo-determinant that distinguishes Dw4 and is it similar to the antibody-binding epitope (s) that have been identified in this study? The phenomenon of alloreactivity and the nature of the allo-determinants have been extensively studied and debated. It is now generally accepted that alloreactive T cells bind complexes of allo-MHC plus a peptide, which may be cellular- or serum-derived. This theory is based on considerable experimental

evidence demonstrating peptide-dependency of alloreactive T cell clones, while there is no clear evidence that empty HLA molecules stimulate alloreactive T-cells (reviewed in Sherman and Chattopadhyay, 1993). Whether or not the bound peptide is part of the allo-determinant or whether it induces a conformational change in the critical part of the alpha helix to which the TCR binds, has not been completely elucidated. For example, some alloreactive T cell clones do not exhibit peptide specificity, since they have been shown to respond to otherwise empty MHC molecules that have been filled with different synthetic peptides.

It is beyond the scope of the data presented in this study to answer the question regarding the composition of the antibody binding epitopes. Unfortunately, an informative cell expressing B1*0408 (86G) was unavailable to test the importance of 86G to the integrity of these epitopes. However, since 86G or 86V is found on all DR molecules and since 71K is also found on the β chains of DR3 and some DR52 molecules, none of which bind either NFLD.D11 or NFLD.D12, it is likely that other residues and/ or peptide contribute to the formation of these allele specific epitopes.

The reactivities of the two antibodies are analogous to the type of reactivity reported for alloreactive T cells. The best example of this is the restoration of NFLD.D11 binding, but not of NFLD.D12 binding to Dw4-expressing mouse cells when these cells are pulsed with crude membranes. This finding is reminiscent of an experiment by Heath et al., (1991) in which a cyanogen-bromide, cleaved murine-derived peptide was used to restore the cytolytic activity of an alloreactive T-cell

clone for H-2K^b molecules that were expressed on a human cell. Furthermore, the failure of both mAbs to bind Dw4 molecules expressed on antigen processing mutants is similar to the failure of alloreactive T cells to bind empty HLA molecules. For example, it is well-known that transfected class II molecules on T2 are conformationally unstable and are largely occupied by a peptide derived from the Invariant chain (Sette et al., 1993); however, they can efficiently present exogenously added peptide to the appropriate antigen-specific T cells. It will therefore be informative to pulse T2-Dw4 and BLS-Dw4 with B-cell derived and other peptides to see if the antibody binding epitopes can be restored.

It is interesting to speculate on the derivation of the peptide that purportedly contributes to these antibody binding epitopes. The fact that the NFLD.D11 binding epitope was restored by the human cell lysate and not by the culture medium that was used for growing the cells suggest that the involved peptide(s) is endogenously-derived. The failure of NFLD.D12 to be restored by this lysate could be due to inadequate processing of the human proteins by the mouse fibroblast. It is also of interest that the NFLD.D12 epitope was inadequately expressed on peripheral B cells, which suggests that the appropriate peptide may be supplied by EBV or possibly by a blast-related antigen. Experiments are planned to test these ideas.

CHAPTER 9

GENERAL SUMMARY AND DISCUSSION

9.0 PREFACE

The work described in this thesis was essentially done in four phases: i) an attempt to make transfectants expressing DR4 molecules using genomic DNA; ii) preparation, selection and cloning of hybridomas secreting mAbs that are specific for DR4 molecules; iii) complete specificity analysis of the anti-DR4 mAbs using various types of cells; iv) epitope mapping studies using transfectants that express mutated DR molecules. Most of the experimental details and results have been summarized and discussed in each of five manuscripts (**Chapters 4-8**) that correlate approximately with the different phases mentioned above. In the ensuing sections I will focus on the background under which certain aspects of the research were carried out and highlight some unanticipated and intriguing findings that have emerged from this work. I will also discuss the significance of the information gained from the epitope mapping studies to furthering our knowledge about a putative "shared epitope" that is carried by the various DR molecules that are associated with Rheumatoid Arthritis.

9.1 CHOICE OF IMMUNOGEN FOR MAKING DR4 MONOCLONAL ANTIBODIES

When this work was initiated there were no known monoclonal antibodies (mAbs) that exclusively recognized either the serologically-defined DR4 specificity, the putative serological splits (DR4.1, 4.2, and 4.3), or any of the biochemical or T-cell defined subtypes. One antibody, NFLD.M1, which was shown to be strongly reactive with DR4 molecules, also reacted weakly with some DR52 positive cells (Drover et al., 1986). In addition a few mAbs were described that recognize some of the DR4 subtypes, but crossreacted with DR1 and/or DR9 (reviewed in Bodmer et al., 1984; Nispersos et al., 1987). These partially resembled the MC1 specificity, which is associated with DR1 and the DR4 subtypes (Dw4, Dw14 and Dw15) and which was shown to confer a higher degree of risk for the development of RA than either DR4 or DR1 alone (Duquesnoy, 1984)

It was clear from previous experience and various reports that the vast majority of mAbs generated using B cells as immunogens were against non-HLA molecules or monomorphic HLA determinants (reviewed in Drover, MSc thesis, 1986). Thus, while the probability of making mAbs to the "shared epitope" associated with RA was viewed optimistically, the challenge in producing allele-specific monoclonal antibodies was fully recognized. The fact that Heyes et al. (1986) had successfully used DP-expressing transfectants to make a mAb to a polymorphic

determinant on DP molecules, which were normally typed by primed-T lymphocytes, was particularly encouraging. It was therefore anticipated that the likelihood of making DR4 allele-specific mAbs would be enormously increased, if DR4-expressing transfectants were available for immunization.

9.2 EFFORTS TO CREATE DR4-EXPRESSING TRANSFECTANTS

Since no DR4-expressing transfectants or cloned DR4 genes were immediately available, an attempt was made to create transfectants using genomic DNA. The impetus to carry out these experiments came from the work of Kavathas and Herzenberg (1983, 1986), who had successfully obtained HLA class I-expressing transfectants by co-transfecting thymidine kinase (tk) negative L cells with uncloned genomic DNA and the chicken tk gene. Based on their calculation that each transformant contained about 1500 Kb of DNA, which is larger than the entire class II region, it was predicted that a class II-expressing transfectant could be made in a similar manner.

Subsequent experiments produced transfectants that expressed either HLA-A or HLA-B genes, but failed to yield stable transfectants that expressed DR4 or any other class II genes. However, the simplicity of the methods that were developed for selecting and isolating these transfectants (immunomagnetism and replicate plating

combined with CELISA, described in **CHAPTER 4**) were considered a notable contribution to this field. In retrospect, it is perhaps not surprising that stable expression of class II genes was not achieved. Most of the DNA that is taken up during transfection is likely degraded and the amount of DNA that encompasses the region encoding a DR molecule is significantly larger than that which encodes a class I heavy chain. Furthermore, class II expression requires that both DRA and DRB genes be properly integrated into the mouse chromatin structure, while class I expression requires the integration of a single class I gene. This is because the resulting heavy chain is able to pair with the endogenous mouse β_2 microglobulin.

Experiments that were planned to use cloned genes and to investigate whether a mouse B cell lymphoma (A20), which constitutively expresses class II, would make a better recipient for class II expression, were terminated because a set of relevant DR-expressing transfectants became available through the generosity of Raffi Sekaly and Robert Karr.

9.3 TRANSFECTANTS VERSUS B CELLS AS IMMUNOGENS FOR MAKING ALLELE-SPECIFIC MONOCLONAL ANTIBODIES

Early in the second phase of this study it became apparent that immunization with DR4-expressing mouse cells was not a panacea for obtaining allele-specific mAbs. The work described in **CHAPTER 5** underscores some of the difficulties that were encountered. For example, it was found that most of the antibodies that were produced to the transfectants were either irrelevant or L-cell specific, which suggested that the L-cells carried tumour-associated antigens and probably bound proteins or peptides that were derived from the culture medium. In the final analysis twenty-nine fusions yielded 15,000 hybrids, of which approximately seventy hybrids, or one in every 200, secreted an antibody to a polymorphic determinant on HLA-DR molecules. Although most of these mAbs bound to polymorphic determinants that were found on a large variety of different DR molecules, several proved to have interesting and/or novel specificities.

To recapitulate, NFLD.D1 recognized a common epitope on all DR4 molecules and no others; NFLD.D7 bound an epitope that is present on all DR4, DR2 and DR52 molecules; several (NFLD.D2, NFLD.D3, NFLD.D4, NFLD.D8, NFLD.D9 and NFLD.D10) bound some of the DR4 subtypes as well as some DR molecules that carried homologous sequences at positions 67-86 on the alpha helix of the DR β chains. One of these (NFLD.D2) was especially exciting because its

binding epitope correlated with that of the "shared epitope" that had been predicted to be implicated in the development of Rheumatoid arthritis (Gregersen et al., 1987).

Paradoxically, after having made such a huge effort to obtain anti-DR4Dw4 mAbs from fusions in which transfectants were used as immunogens, the most interesting mAbs came from a fusion in which the mouse was immunized with B cells. These two mAbs (NFLD.D11 and NFLD.D12, described in **CHAPTER 8**), which have the ability to differentiate the Dw4 subtype from all other subtypes, were created through serendipity. The mouse from which they were derived had first been immunized with DP molecules, but was boosted with a Dw4-positive human BCL prior to fusion. Dw4 molecules could potentially have been present in the primary immunization, if the DP monomorphic mAb (NFLD.M67), which was used to isolate the DP molecules, binds an epitope on the DP α chain; in this way mixed molecules (eg. DPα DRβ1*0401) as well as normal DP molecules may have been isolated. However, it is more likely that these Dw4-specific mAbs mAbs, which are IgM, resulted from the IV boost that acted as a primary immunization for the mouse with respect to Dw4 molecules. This idea is supported by recent experiments, in which fusions done after primary immunizations, produced an array of interesting anti-HLA mAbs (unpublished data). Thus, the work has come full circle as experiments are now in progress to produce anti-Dw14 mAbs using a similar immunization scheme.

9.4 NFLD.D MABS: COMPARISON TO OTHER MONOCLONAL ANTIBODIES

As previously mentioned, the number of mAbs available that identified polymorphic determinants, particularly those restricted to a particular serotype or subtype, were disappointingly small. It was therefore of some interest to see if mAbs produced during the course of this work, particularly those made using transfectants, had novel specificities. Since this work coincided with the 11th IHW, the opportunity was provided to compare these specificities with consensus specificities for other DR4-specific mAbs and alloantisera (Juji et al., 1992; Marsh et al; 1992) or antibodies that have been otherwise described.

The DR4-specific mAb, NFLD.D1, was found to have an identical reactivity pattern to another mAb, GS359-13F10 (Alber et al., 1989); however, it was found to map to a different region of the second domain of DRβ*04 chains (Maurer and Gorski, 1990 and **CHAPTER 6**). A similar mAb (AMO 801, 5G616E10) was analyzed in the serology component of the 1991 IHW, but it was found to have extra reactivity with DR16-positive B cells. The pan-DR4 specific mAb, NFLD.D7, which also binds DR52 and DR2 molecules is somewhat similar to OLI 823. A subtle difference, however, is that OLI 823 but not NFLD.D7, reacts with DR8 positive cells (Juji et al., 1992). Whether or not they map to the same region of DR4 is not known as there is no mapping data available for OLI 823..

Several mAbs including N1 (BRS 801), PLM 14 (MAZ 801), AC1.59 and JSI

appear similar to NFLD.D10, since they all bind DR4 molecules with the exception of the Dw10 subtype and also various non-DR4 subtypes. For example, N1, a human mAb originally described by Yendle et al., (1990), appeared to be identical to NFLD.D10, since both bind to B cell lines that express DR1, DR2, DR14, DR9 and DR10. However, analysis of N1 in the 11th IHW showed that it was essentially negative with DR2-positive peripheral blood B cells; in contrast NFLD.D10 binds strongly to DR2-positive cells BCL, B-PBL and transfectants. The other mAbs also had fine differences in their specificities; for example, MAE 801 (PLM14) does not bind DR2-positive cells (Juji et al., 1992); AC1.59 does not bind DR2, but crossreacts with some DR8 and DR5-positive cells (Richardi et al., 1984); JSI does not bind DR9, DR10 or DR2, but binds DR3 and DR52 (Sachs et al., 1986).

Each of the other mAbs (NFLD.D2, NFLD.D3, and NFLD.D8) has subtle differences from each other, but clearer differences from NFLD.D10 or any of the mAbs described above. In particular, they react either weakly or negatively with the DR4 subtypes Dw13 and DwKT2. NFLD.D2 closely resembles the MC1 specificity (Duquesnoy, et al., 1984) and the mAb, CC CL 20 (Dejelo et al., 1986), but again each has a somewhat different reactivity pattern. For example, NFLD.D2 additionally reacts with a DR51 subset of molecules encoded by DRB5*02 genes, while CC CL 20 binds poorly to Dw4 subtypes.

The singularity of NFLD.D11 and NFLD.D12, which were made using BCL as immunogens, has previously been addressed (CHAPTER 8). As far as can be ascertained from a review of the literature, NFLD.D11 is the only known antibody which can differentiate Dw4 from closely related DR4 and all non-DR4 molecules, while NFLD.D12 is similar, but crossreacts with Dw14. The only other mAb reported to recognize subtypic differences in the DR4 specificity is 6ED, which was made using a Dw13 synthetic peptide (DRB1:66-81) (Muller et al., 1992). This mAb preferentially binds Dw13 and DwKT subtypes but was found to give inconsistent reactions with Dw4 and DR2 positive cells.

9.5 LOCATION AND COMPOSITION OF POLYMORPHIC ANTIBODY-BINDING EPITOPES ON DR4 MOLECULES

The availability of completely HLA-genotyped BCL (Yang et al, 1989; Kimura et al., 1992) and transfectants expressing single HLA specificities (Inoko et al., 1992) combined with sequence data for all the known class II genes (Marsh and Bodmer, 1992) have made it possible to assign exact specificities to mAbs. Although the epitopes for some mAbs can be correctly predicted from such information, precise epitope mapping is ideally done using cells that express appropriately-mutated genes. Through the generosity of Robert Karr, a set of transfectants that expressed mutated

DR genes was made available for detailed mapping studies. Thus, mAb binding epitopes were identified on the second domain, the alpha helix of the peptide binding site and to the first and second hypervariable regions of the DR4 β chain. For some of these epitopes, peptide-binding residues in the groove were involved (see section 9.6).

9.5.1 Epitopes on the Second Domain

The epitope for NFLD.D1, which is exclusively found on DR4 molecules did not map to the DR4-specific residue (DRB1:96:Y), which was previously shown to be critical for the DR4-exclusive mAb, GS359-13F10 (Maurer and Gorski, 1991). However, it did map to the β_2 domain, which appears to contain another allele-specific residue, B1:180:L (Andersson et al., 1987; Gorski, 1990; Marsh and Bodmer, 1992). Studies are currently being done in Robert Karr's laboratory to test whether this position is critical to the formation of the NFLD.D1 epitope.

It is interesting that the only DR4-exclusive mAbs, NFLD.D1 and GS59-13F10, thus far identified, map to different regions of the second domain. Moreover, a third mAb, NFLD.M1, which is essentially DR4-specific with B-PBL (unpublished data), but is weakly reactive with DR52 positive BCL and transfectants, also binds an epitope in the β_2 domain (Alber et al., 1989). We previously suggested that DRB:140T is critical for this epitope (Alber et al., 1989), but during this study, it was

shown that NFLD.M1 bound all three different DR52 subtypes (**Figure 6.1**), of which only one subtype carries 140:T. Thus, although the location of the NFLD.M1 epitope is known, the residues which contribute to its formation are not immediately apparent and will require further studies.

It may be of some significance that so few amino acids dispersed over the second domain constitute several different epitopes. There is some evidence to suggest that these amino acid differences can also induce structural alterations in T-cell defined determinants, which are clustered around the peptide binding site. For example, the only difference between the two T-cell defined subtypes of DR7 (Dw7 and DB1) is an arginine replacement for a glutamine at position 107. Furthermore, in the murine class I system, it has been shown that mutations in the α_3 domain of a class I molecule (equivalent to β_2) altered the binding of alloreactive T cells and antibody (Potter et al., 1987; Maziarz et al., 1988). Since the accessory molecules, CD8 and CD4, interact with the class I α_3 domain and class II β_2 respectively, one might speculate that the polymorphisms in these domains may somehow affect the interaction of MHC and these accessory molecules.

9.5.2 Epitopes that Map to Sequences on the Floor of the Peptide Binding Site

There was no evidence from these mapping experiments to suggest that the DR4 allele-exclusive residues 13H and 33H, which are located on the floor of the peptide binding groove (Brown et al., 1993), were involved in the DR4-exclusive mAb epitopes described above. The side chains of these residues are buried by bound peptide so that they are not available for direct interaction with antibody. However, sequences on the floor were shown to be important for the NFLD.D7 binding epitope, which was lost when the first and second hypervariable regions of DRB1*04 were replaced with those from DRB1*07 **(Figure 6.3)**. Subsequent analysis of NFLD.D7 using DRB1*0401 mutants, which expressed single amino acid substitutions in these regions (9E-W, 11V-G, 13H-Y, 28D-E and 30Y-L), showed no altered binding to any of these mutants (data not shown). It is suggested that polymorphic residues on the floor of the groove may induce conformational changes in remote structural determinants or perhaps, they induce conformational changes through the family of peptides that they bind **(see section 9.6)**.

9.5.3 Epitopes on the Alpha Helix of the Peptide Binding Site

Most antibodies that bind all DR4 subtypes with the exclusion of Dw10, usually bind other DR molecules that have glutamine (Q) at position β70. As predicted this residue was shown to be critical for NFLD.D2, NFLD.D3, NFLD.D8 and NFLD.D10 binding (**CHAPTER 7**); it has also been shown to be critical for the binding of similar antibodies, CC CL 20 (Hiriawa et al. 1991) and N1 (Barber et al, 1991). However, none of these mAbs bind all DRB:70Q positive molecules, which suggests that many diverse epitopes are generated from a common sequence in which position 70 is pivotal. Furthermore subtle epitope variations result from differences in peptide binding residues in these MHC grooves. The information gained from epitope mapping with the DR mutants and natural variants indicate that the footprints of these mAb-binding epitopes on DRB*0401 are composed as follows: NFLD.D2, DRB1*67-74:LLEQKRRAA/86:G/28:D; NFLD.D3,B1:70-74:QKRAA/28:D/30Y; NFLD.D8, B1: 67-74:LLEQKRAA; NFLD.D10, B1:70-73:QKRA. It is important to note that these residues may not be all inclusive; for example, the pattern of binding to non-DR4 wild type molecules (**Table 7.1**) suggest that other residues on the floor, (for example, those at positions 26, 31, 32 and 33) may modify the epitopes of NFLD.D2, NFLD.D3 and NFLD.D8. However, the appropriate mutants were not available to test this hypothesis.

The epitopes for NFLD.D11 and NFLD.D12 are predicted to involve

DRB1:71K and 86G, since these are the only two residues that differentiate the Dw4 subtype from Dw14. However, as previously noted, it is unlikely that these antibodies directly contact these residues since their side chains are not solvent exposed (Brown et al., 1993). Furthermore, these residues are also found on other DR molecules, to which NFLD.D11 and NFLD.D12 do not bind. As discussed below, it is predicted that the Dw4-specific mAbs bind either a composite of residues and peptide or a conformational determinant that is extremely sensitive to peptide in the groove.

9.6 PEPTIDE-DEPENDENT, ANTIBODY-BINDING EPITOPES ON DR MOLECULES

The fact that the reactivity patterns for several mAbs differed according to the cell type on which the DR molecules were expressed was intriguing. Two mAbs (NFLD.D1 and NFLD.D7) that were made using transfectants as immunogens generally bound well to transfectants, but unexpectedly, most of the other mAbs, reacted better with BCL than with the relevant transfectants. Antibodies which mapped to the putative RA susceptibility sequence were particularly sensitive to whether the DR molecules were expressed on mouse or human cells. The most conspicuous example was NFLD.D2, which consistently reacted strongly with Dw16-positive BCL, but negatively with Dw16-positive transfectants; **(Figure 7.1 and unpublished data)**. NFLD.D2 also reacted weakly with Dw13, KT, and DR15-

positive BCL, but not with the relevant transfectants or normal B-PBL. Similarly, NFLD.D3 and NFLD.D8 also showed some differential binding to human and mouse cells expressing these DR molecules., but importantly, they bound Dw16-positive BCL and transfectants equivalently.

In considering the reasons for this phenomenon, it was duly noted that other HLA antibodies were reported to have a broader reactivity pattern with BCL than they do with normal peripheral blood B cells or mouse cell transfectants that express the same specificities (Stinchcombe et al., 1985; Yendle et al., 1990). It is possible that the higher density of HLA molecules on BCL (Trucco et al., 1980) or low antibody affinity for a particular DR specificity may explain some, but not all examples of differential binding. As previously discussed in **CHAPTERS 6 AND 7,** the creation of artifacts due to differences in antibody affinity or expression of DR molecules should have been eliminated or reduced by expressing binding of the test mAbs relative to a DR positive control mAb.

The explanation that is favoured and for which there is supporting evidence, is that differences in peptides that bind to the grooves of these DR molecule affect the conformation of the antibody-binding epitopes. For example, the epitopes for these mAbs require a contribution from amino acids (β28, 74 and 86) that are involved in different peptide binding pockets in the MHC groove (Brown et al., 1993). Importantly, the same epitopes were profoundly altered on Dw4 molecules expressed on antigen processing mutants. The MHC-grooves of molecules expressed

on these cells are either empty or obscured by a large invariant chain fragment that has not disassociated (Riberty and Cresswell, 1992; Sette et al., 1993). Such molecules have previously been shown to be non-reactive with certain antibodies to polymorphic determinants (Mellins et al., 1991). Interestingly, one of the first antigen-processing mutants identified was produced by immunoselection with a peptide-sensitive DR3-specific mAb (Mellins et al., 1990, 1991; Sette et al.,1993).

Sequencing of peptides eluted from class II grooves has revealed that the majority of these peptides are endogenously derived (Rudensky et al., 1991; Chicz et al., 1993). Therefore it is likely that the array of peptides that bind to the MHC grooves of molecules expressed on EBV-transformed B cells, normal B cells and mouse cell transfectants will be different in composition, size and orientation. Such differences have the potential to alter conformational determinants on the alpha helix, as was observed for the antibody-binding epitopes for which DRB1:70:Q was critical. Such antibodies are often referred to as "peptide-sensitive".

At the extreme end of this spectrum of peptide-sensitive antibodies are the Dw4-specific mAbs (NFLD.D11 and NFLD.D12) which absolutely require particular peptides or subsets of peptides in the groove for binding. Since they both exhibit no binding to Dw4-positive mouse cells but differ in their binding to B-PBL, it seems unlikely that the required peptides are derived from culture medium. The data presented in (**CHAPTER 8**) suggests that the peptides are endogenously-derived; furthermore, the lack of NFLD.D12 binding to normal B-PBL suggests that the

peptide involved in this epitope may be EBV-derived. Further investigation is required and experiments are planned to elucidate the nature of the peptides involved in these epitopes (**See section 9.9**)

9.7 SIMILARITIES BETWEEN THE SPECIFICITIES OF ALLOREACTIVE T CELLS AND ALLELE-SPECIFIC ANTIBODIES

The generally accepted paradigm for antibody recognition of allo-MHC is that an antibody binds to conformational determinants and that its affinity is such that it is relatively unaffected by subtle changes to the conformation that may be brought about by the array of different peptides that bind to the groove. In contrast, alloreactive T cells are believed to bind either a composite of peptide and MHC or are exquisitely sensitive to peptide-induced alteration of critical determinants on the alpha helix (Sherman et al., 1993; Ramansee et al., 1993). Thus, to suggest that certain antibodies potentially recognize allo-determinants in the same way as T cells may seem at first like a renunciation of the current dogma of T-cell and antibody-recognition of antigen. However, it has been clearly shown in the murine system that the binding of certain allo-antibodies to H-2 is peptide-dependent.

Substantial evidence for peptide involvement in antibody-recognition of allo-epitopes has come from two recent reports regarding several allo-antibodies to H-2K^b. Both studies utilized the availability of mutant cell transfectants which do not

normally express class I molecules due to deletion of the TAP1 genes; however by utilizing temperature shifts, such cells can be induced to express empty class I molecules. Such molecules are useful for identifying the composition of peptides that affect T cell or antibody allo-recognition. Thus, some allo-antibodies to H-2K^b were shown to differentially bind to different peptide/H-2K^b complexes (Hogquist et al., 1993; Sherman et al., 1993). Furthermore, these mAbs varied in their ability to immunoprecipitate H-2K^b molecules that were loaded with known peptides (self or foreign). Using different variants of a peptide that had been shown to affect the binding of some but not all antibodies, Hogquist further showed that the sensitivity of certain antibodies to peptides was due to steric hinderance induced by solvent-exposed residues on the peptide. Importantly, a few antibodies were sensitive to peptide variants in which the residues were not exposed but buried in the MHC groove. It was further shown by Sherman et al (1993) that acid-eluted peptides from K^b molecules immunoprecipitated with these antibodies, varied in their ability to reconstitute the activity of a panel of T cell clones.

It is intriguing that the reaction patterns of the peptide-sensitive or peptide-dependent anti-DR4 antibodies that are described here, have similar or identical reaction patterns to some of the specificities of alloreactive T cells (Goronzy and Weyand, 1989, Flomenberg et al, 1989; Obata et al., 1992). These can be divided into two groups: allele-exclusive mAbs such as the anti-Dw4 (NFLD.D11) which, like an alloreactive T cell has the power to differentiate Dw4 from Dw14. The second group

is crossreactive and recognizes determinants that are formed by homologous sequences on the alpha helix. It is thus interesting that, like antibodies, more alloreative T cell clones belong in this category than in the former category. For example, several T cell clones recognize different but overlapping epitopes on DR4 and certain non-DR4 molecules. One such T cell clone (6052) from the 10th IHW recognizes Dw4, Dw14, Dw1, Dw16 molecules; similar clones have also been described by others (Goronzy and Weyand, 1989). Interestingly, this pattern is almost identical to that of NFLD.D2 except that the latter also binds DRB5*02-encoded molecules on DR16 haplotypes. Again it is interesting to note that another clone (6047) from the same workshop recognized a determinant on cells expressing DR1, DR16, Dw14 and Dw16, which is similar to the NFLD.D3 specificity. It has been clearly demonstrated, using site-directed mutagenesis of the relevant DR genes, that the CC CL 20 antibody-binding epitope is remarkably similar to a T cell determinant on DR1, Dw16 and Dw14 molecules (Hiriawa et al., 1991).

Altogether these observations and results suggest that alloreactive T cell clones and certain antibodies bind a similar set of allo-determinants. It is also noteworthy that some investigators have shown that some murine allo-antibodies showed a total dependence on a particular subset of peptides in the groove. For example, Murphy et al (1990) reported on a mAb that recognized a subset of I-A^b molecules, which were later shown to contain peptides from the I-E α chain (Rudensky et al., 1991).

9.8 SIGNIFICANCE OF NFLD MABS FOR FUTURE STUDIES OF THE HLA-DR ASSOCIATION WITH RHEUMATOID ARTHRITIS

The HLA-DR association with Rheumatoid Arthritis was extensively reviewed in **CHAPTER 2.** In summary a homologous sequence DRB1:70-74:QKRAA/QRRAA/RRRAA is found in approximately 90% of hospital-based patients. These sequences are predicted to form similar epitopes, usually referred to as "shared epitopes" that somehow confer susceptibility to the development of RA. However RA is heterogenous with symptoms ranging from mild polyarthritis to debilitating destruction of the joints and even to showing extraarticular features. An accumulating body of evidence suggests that the severe forms of this disease are more likely to occur in DR4-positive individuals. In particular, the genotype, Dw14Dw4 confers the highest degree of risk for severe forms of the disease (Wordsworth et al., 1992; Weyand et al., 1992). It has therefore been suggested that these DR subtypes may serve as useful prognostic markers, but more studies of a prospective nature are required to confirm this hypothesis. Possible roles for NFLD antibodies in such studies are discussed below.

9.8.1 Application of anti-DR4 mAbs for Diagnosis and Prognosis

At least three of the mAbs that are described here may be useful in aiding the diagnosis and treatment of RA. Firstly NFLD.D1 can identify all DR4 individuals; NFLD.D2 can identify those that carry the putative susceptibility sequence; NFLD.D11 can identify individuals who have Dw4, which is the subtype most commonly associated with severe forms of RA. Other mAbs which map to this region may also be useful for identifying susceptible individuals. In fact one of the objectives of this work was to study the binding of mAbs such as these so as to compare results on a panel of cells from RA patients with those on cells from normal controls.

Preliminary studies have been carried out on a panel of EBV-transformed cells and B-PBL from patients and controls. Essentially, the mAbs gave the same binding pattern on RA cells as they do on cells from healthy people, that is the binding correlated with the same DR specificities. Studies are in progress to test the usefulness of NFLD.D2 and NFLD.D11 for identifying RA patients who carry the susceptibility sequence and those who may be at an increased risk for the development of severe RA.

9.8.2 Relevance of Antibody and Epitope Mapping Studies to Understanding the Nature of the "Shared Epitope" in the Development of RA

The epitope mapping studies described here provide direct experimental evidence for the existence of several overlapping structural determinants on the alpha helix of the DR molecules that are associated with RA. This concurs with what was found for T cell recognition of determinants that map to this region (Goronzy and Weyand, 1989; Hiraiwa et al., 1991). A significant finding from these studies was that the NFLD.D2 binding epitope, which most closely resembles the putative RA"shared epitope", was profoundly modified by the DR framework and peptide in the groove. By implication the conformation of the RA "susceptibility epitope" will vary also on the different DR molecules that are associated with RA, and may explain why non-DR4 molecules confer lower relative risks for the development of RA, despite carrying an identical susceptibility sequence.

9.8.3 Putative Role for DRB1:28D in the RA Susceptibility Sequence

An in-depth analysis of these data suggest that position 28 may be intimately involved in conferring susceptibility to the development of RA. The evidence for this is derived from several observations. First, all non-DR4 molecules that carry the RA susceptibility sequence, but confer low relative risk for the disease, have glutamic

acid at position 28. In contrast the DR4 molecules that carry the same susceptibility sequence and confer the highest degree of risk have aspartic acid at this position. Secondly and importantly, the DRB1:28:D→E substitution in the DRB1*0401 mutant and in natural variants (DR1 & DR14) profoundly altered the NFLD.D2 epitope. Thirdly, because β28 is spatially proximate to the consensus sequence β70-74:QK/RRAA, and because of its importance, along with β71 and β74 in a peptide-binding pocket, a substitution at this position could have serious implications for the binding and/or orientation of an arthritogenic peptide(s) by these molecules. In these experiments peptide in the groove clearly altered the structure of the NFLD.D2 binding epitope.

It is therefore predicted that either the substitution at position 28, itself, and/or peptide binding modifies the "shared epitope" such that the epitope on DR4 molecules is conformationally different from that which is present on DR1 molecules. Whether it is an alteration of the conformation of the shared epitope or the ability to bind different peptides in the groove that is responsible for the different degrees of risk conferred by these different molecules is open to speculation.

9.9 FUTURE PLANS

The potential of these mAbs as markers for RA susceptibility and severity have already been addressed in section 9.8. Since a Dw14-specific mAb would increase the potential of their value as prognostic markers, efforts will be made over the next few months to accomplish this. It is further anticipated that these mAbs may be useful for probing the nature of the peptides that bind to the RA-associated MHC grooves and those peptides that are involved in the composition of allodeterminants on the Dw4 molecules.

9.9.1 Purification of MHC-Peptide Complexes from Rheumatoid Synovium

If one operates on the premise that RA is initiated or persists due to T cell recognition of an "arthritogeneic" peptide bound to the groove of a "susceptible" DR molecule, then it is conceivable that, by using these mAbs for the purification of MHC-peptide complexes, information may be gained about the nature of the "arthritogeneic" peptide. The advantage of this collection of mAbs over monomorphic mAbs is that they are specific for the molecules that are implicated in RA and that they include antibodies whose epitopes are either sensitive to or dependent on the peptides that are present in the grooves. For example, NFLD.D2 preferentially binds DR molecules which are implicated in susceptibility to RA and

is peptide senstive; NFLD.D11 is Dw4-specific and peptide-dependent; NFLD.D1 is DR4-specific and appears to be unaffected by peptides that are bound to the grooves. Thus, the application of these mAbs to the purification of MHC-peptide complexes from RA synovium and microsequencing of the peptides, might provide insight into the nature of the peptide that triggers RA. Such findings might have implications for the design of therapeutics.

9.9.2 Mapping of Dw4-specific Epitopes

The composition of the epitopes for the two Dw4-specific mAbs is particularly intriguing and requires further characterization. Preliminary work using crude membranes from EBV-transformed cells indicated that at least the NFLD.D11 binding epitope could be reconstituted on Dw4-positive mouse L cells. The experiment needs to be repeated using peptides from cyanogen-bromide cleaved membranes (Heath et al., 1991). These peptides could be fractionated by HPLC and each fraction tested to see if one or more can reconstitute the epitopes on Dw4-positive L cells and/or Dw4 positive T2 cells. This is analogous to similar work described by Sherman et al., (1993) on reconstitution of alloantibody epitopes and alloreactive T cell determinants on the empty H-2K^b molecules expressed by the T2 antigen processing mutant cell line. Restoration of these epitopes on Dw4-positive L cells would be of particular interest, since the panel of DRB1*0401 mutants

(described in **CHAPTER 7**), in which each carries a single amino acid substitution, could be used to investigate the importance of residues on the floor and the alpha helix to the formation of these determinants.

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