STUDY OF A MONOCLONAL ANTIBODY TO HUMAN B CELLS

MARY SHEILA LEWIS DROVER

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BY

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Department of Medicine
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ABSTRACT

The purpose of this work was to characterize and determine the specificity of a mouse monoclonal antibody (NFLD.M1) which was derived from a fusion between SP2/0-Ag14 and spleen cells from a Balb/c mouse that had been hyperimmunized with B-cells from a chronic lymphatic leukemic patient. Cloning was done by limiting dilution and positive clones were selected by screening on a panel of viable cells using the cellular enzyme-linked immunosorbent assay (CELISA). This assay was shown to be more specific and sensitive than an ELISA that used glutaraldehyde-fixed cells.

Two sources of the antibody (purified IgG1 from ascites fluid and supernatant from overgrown cultures) appeared to be identical in their serological pattern on several B-cell lines. Specificity testing using the CELISA and several different cell types revealed that NFLD.M1 recognized some B-cells, but failed to react with any of the T-cells tested. A Frequency Distribution plot of the data showed that NFLD.M1 reacted with the cells in a bimodal fashion compared to the normal distribution observed with the monomorphic monoclonal antibody, NEI anti-Ia. Furthermore when NFLD.M1 antibody was expressed as a percent of the NEI anti-Ia it was found that all the DR4 positive cells produced values greater than 50% whereas DR4 negative cells gave values less than 30%. Using

30% as a cutoff point a correlation analysis was done on the CELISA results for 42 cell lines. The r value obtained for DR4 and NFLD.M1 was 1 with a p value of 2×10^{-10}. In addition significant r values were obtained for DRw53 and DQw3 which are in linkage disequilibrium with DR4.

One-dimensional electrophoresis of the immunoprecipitated molecules from a DR4 cell produced a banding pattern that was compatible with that of the alpha and beta subunits of DR.

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List of Abbreviations

BB	Blocking buffer
B-LCL	B-lymphoblastoid cell lines
BSA	Bovine serum albumin
CDC	Complement dependent cytoxicity
CELISA	Cellular enzyme-linked immunosorbent assay
CLD	Cloning by limiting dilution
CLL	Chronic lymphatic leukemia
CM	Cloning medium
dH_2O	Distilled water
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FH	Ficoll-Hypaque
GA	Glutaraldehyde
GA-F	Glutaraldehyde-fixed
GAM	Goat anti-mouse
HAT	Hypoxanthanine-aminopterin-thymidine
HM	Hybridoma medium
HRP	Horse radish peróxidase
HT	Hypoxanthanine-thymidine
LCL	Lymphoblastoid cell lines
LN_2	Liquid nitrogen
MCAB	Monoclonal antibody

PA	Protein A
PBM	Peripheral blood mononuclear cells
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline containing 0.05% Tween-20
PLL	Poly-l-Lysine
PVC	Poly vinyl chloride
RAM	Rabbit anti-mouse
RT	Room temperature
s	Standard deviation
SAS	Saturated ammonium sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T-LCL	T-lymphoblastoid cell lines
$\bar{X}$	Statistical mean

CHAPTER I INTRODUCTION

The work described in this thesis deals mainly with the characterization of a monoclonal antibody (NFLD.M1) which was produced in this laboratory, and the selection and application of an appropriate assay to detect and characterize its specificity. Preliminary screening of supernatant from the hybridoma culture had indicated that the antibody was recognizing a B-cell polymorphism. Specificity testing using antibody from the cloned hybridoma suggests that NFLD.M1 is directed to a determinant on HLA-DR4 molecules.

In this chapter some of the relevant literature on new developments in the HLA field, particularly the role played by monoclonal antibodies in increasing our knowledge of this complex system, will be reviewed. Emphasis will be placed on HLA-D region molecules, particularly DR4 and the closely associated molecules, DRw53 and DQw3. In addition methods used to identify and study the specificity of monoclonal antibodies to HLA structures will be reviewed.

1.0 Overview of the Major Histocompatibility Complex

The HLA molecules form a complex system of cell surface glycoproteins that are encoded by multiple genes in

the major histocompatibility complex (MHC) on the short arm of chromosome 6 in man (Figure 1). There are three main sets of closely related genes which are co-dominantly expressed and the products of these genes play important roles in the immune response.

The class I genes encode HLA-A, C and B specificities of which there are at least 23, 8, and 47 allelic variants, respectively. Each molecule is composed of two chains. The heavy chain, alpha (Mr = 43,000) carries the alloantigenic determinants in its most external domain (alpha 3). It is anchored in the membrane and non-covalently associated with a light chain, beta$_2$ microglobin (Mr = 12,000) which is not membrane-bound but is required for expression of HLA-ACB specificities. Beta$_2$ microglobin is encoded on chromosome 15 whereas the heavy chain is encoded in the MHC on chromosome 6.

In addition there are some poorly defined class I molecules that are structurally similar to HLA-ACB and are homologous to the murine Qa/TL molecules. Class I molecules are expressed on all nucleated cells and on platelets. They are mainly involved in antigen presentation to cytotoxic T-cells.

The HLA-D region or class II genes are considerably more complex than the class I genes. There are at least three sub-regions (DP, DQ and DR) each with multiple genes, many of which are known to code for functional proteins. The gene

MAJOR HISTOCOMPATIBILITY COMPLEX

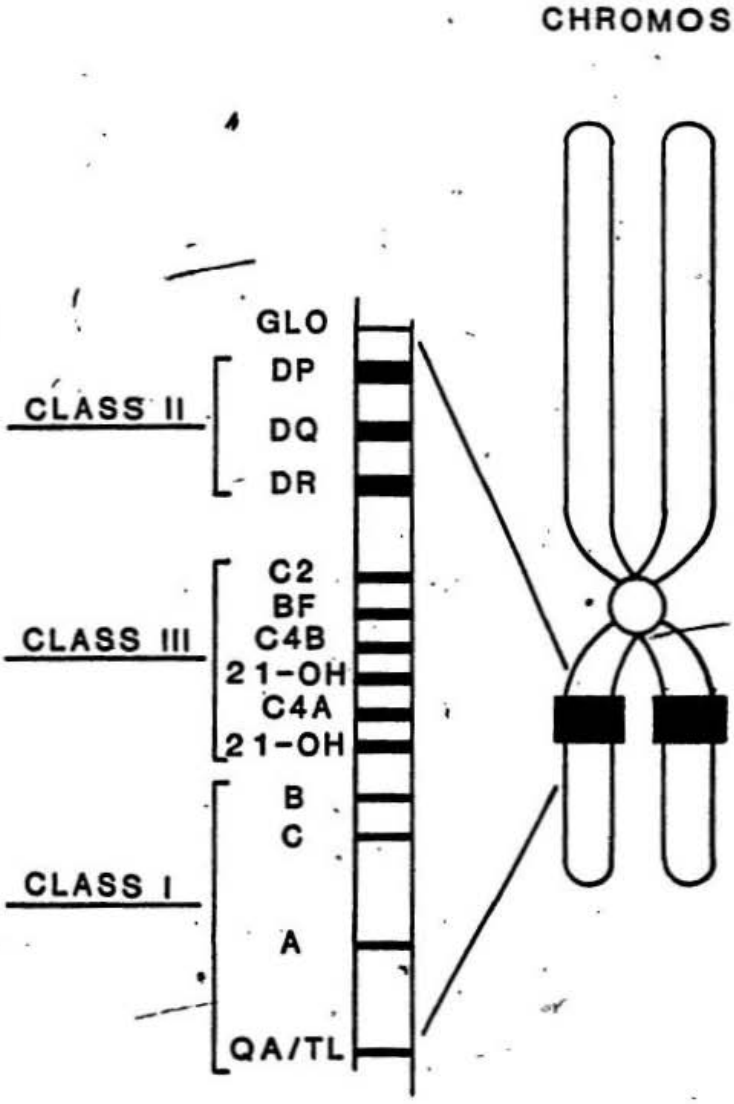

Figure 1. Map of the major histocompatibility complex on chromosome 6 showing the HLA and complement genes; GLO, glycolyase; 21-OH, 21-hydroxylase.

products have been more difficult to define serologically than the class I molecules and much of our present knowledge has come from subregion-specific monoclonal antibodies and from biochemical characterization and DNA-technology (reviewed by Giles and Capra 1985; Moller et al. 1985; Bodmer et al. 1984a; Korman et al. 1985; Trowsdale et al. 1985). The class II molecules are important in antigen presentation to T-helper and inducer cells and are mainly expressed on B-lymphocytes and other antigen-presenting cells such as dendritic macrophages. Although they are not present on resting T-cells, expression can be induced by antigen, mitogen, and γ-interferon stimulation.

The class III genes code for the complement components, C2, Factor B, C4A and C4B, all of which are polymorphic with the C4 components having the most allelic variants. The role played by these components in the immune response with respect to B-cell regulation and disease-susceptibility is an area of current investigation.

1.1.1 Class II molecules

The early work on the identification and characterization of the HLA-D region specificities has been reviewed by several authors including Bodmer (1977) and Winchester and Kunkel (1979). The discovery by Bain et al. (1964) that allogenic cells from unrelated individuals and from dizygotic

twins, but not from monozygotic twins, stimulated each other in a mixed leukocyte culture (MLC) suggested an association with transplantation antigens. The work of Bach and Hirschorn (1964) and Bach and Amos (1967) strongly suggested that these cellularly-defined antigens were controlled by a histocompatibility locus. It was not until some years later that the genes determining these responses were shown to be linked to HLA (van Leeuwen et al. 1971; Yunis and Amos 1973; van Rood et al. 1976) and that there were serological equivalents of the cellularly-defined antigens (HLA-D).

A modification of the MLC technique using homozygous typing cells (HTC) from known HLA-D identical donors has since been used to define 19 variants (Grosse-Wilde et al. 1984). These variants (HLA-Dw) generally correlate with the serologically-defined DR (D-related) specificities which were officially recognized at the 1977 International Histocompatibility Workshop. However, Dw variants do not always correlate with the DR specificity, particularly in the case of Dw4 and DR4. Some of the discordance has recently been explained by the presence of different subtypes within a DR specificity. The contribution of other D-region products such as DQ to MLC-defined Dw specificities still requires clarification and will be discussed in Chapter IV.

The DP (previously named SB) subregion which encodes at least six allelic variants was first described by Shaw et al. (1980) using secondary stimulation of the MLC. Identifi-

cation of DP specificities has not been feasible with conventional antisera since such antisera are rare. Monoclonal antibodies specific for DP molecules have recently been reported (Nadler et al. 1981; Watson et al. 1983; Hurley et al. 1984).

In addition to DR, there are other serologically-defined specificities including DQ previously called MB, DC and DS (Duquesnoy et al. 1979; Tosi et al. 1978; Goyert et al. 1982) and the supertypic DR specificities DR52 and DRw53, previously called MT and BR (Park et al. 1980; Tanigaki et al. 1983). The DQ specificities show allelic association with the DR specificities (Table 1) which has made identification difficult since the alloantisera used for this purpose often contain a mixture of antibodies. Despite this drawback of alloantibodies fourteen and three serological variants were described, for DR and DQ respectively during the Ninth International Histocompatibility Workshop (Bodmer et al. 1984a).

1.1.1A. Structure at the protein level

The structure of the class II molecules (Figure 2) has been revealed by one and two dimensional electrophoresis of immunoprecipitated molecules as well as by amino acid sequencing and more recently by nucleotide sequencing (reviewed in Shackelford et al. 1982; Giles and Capra 1985). DR, DQ, and DP each consists of heterodimers that are

Table 1. HLA-D region serologically-defined specificities in linkage disequilibrium.

DR	DR SPLITS	DR ASSOC	DQ
1	ND	ND	w1
2	LONG	ND	w1
	SHORT	ND	w1
w10	ND	ND	w1
w6	w13	w52	w1
	w14	w52	w1
w8	?	w52	-
5	w11	w52	w3
	w12	w52	w3
3	ND	w52	w2
7	ND	w53	w2
4	4.1	w53	w3
	4.2	w53	w3
	4.3	w53	w3
w9	ND	w53	w3

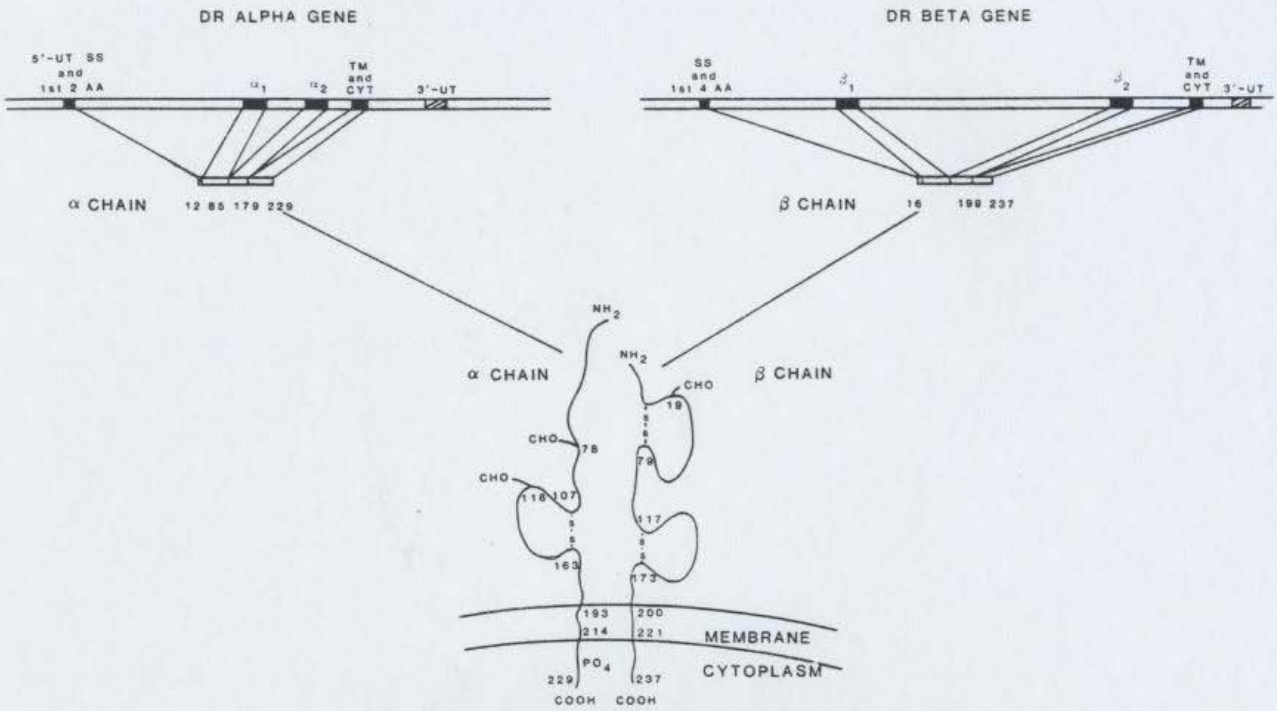

Figure 2. A schematic representation of the arrangement of exons that encode the domains found in the polypeptide structure of the alpha and beta chains of class II molecules (DR shown here). UT, untranslated; SS, signal sequence; TM, transmembrane; CYT, cytoplasmic. $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$, external domains of the alpha and beta chains respectively. (adapted from Stites et al. 1984)

formed by two non-covalently linked glycosylated chains that traverse the plasma membrane (Strominger et al. 1975; Walsh and Crumpton 1977; Springer et al. 1977; Owen et al. 1981; Kvist et al. 1982). Each subunit has a light chain, beta (β) and a heavy chain, alpha (α), with Mr ranging from 27,000-29,000 and 31,000-34,000 respectively (Figure 2). In addition there is an invariant chain, called gamma, Mr = 31,000 (Jones et al. 1979). Although its function has not been elucidated, it appears to be important in transferring the alpha-beta dimer from the endoplasmic reticulum and inserting it into the plasma membrane (Kvist et al. 1982). During transport some of the gamma chains become inserted in a reversed orientation with the carboxyl terminus on the plasma membrane side and the amino terminus on the cytoplasmic side (Claesson-Welsh et al. 1984).

Like most transmembrane glycoproteins the carboxyl termini of the alpha and beta chains are located on the cytoplasmic side of the membrane with the hydrophobic segments traversing it and the amino termini on the external side of the membrane (Kaufman and Strominger 1979; Korman et al. 1982). The DR alpha chain is composed of 229 amino acids (15 internally, 23 transmembrane, and 191 externally), whereas the beta chain has 237 residues (16 internally, 22 transmembrane, and 199 externally) (Kratzin et al. 1981; Yang et al. 1982; Kaufman and Strominger 1982). Both have domain-like structures with the beta chain having two immuno-

globulin-like intrachain disulphide bonds whereas the alpha chain has only one immunoglobulin-like domain (Kaufman and Strominger 1982).

Molecular studies using sequences derived from cloned cDNA and from genomic DNA have shown that each of the domains seen in the protein structure is encoded by a separate gene segment (Figure 2). Most of the polymorphism resides on the the most external domain ($_1$) of the DR beta chains while the alpha chain is invariant (Charron and McDevitt 1979; Shackelford et al. 1982). However DQ alpha chains as well as DQ beta chains have been shown to be polymorphic (de Kretser et al. 1983; Corte et al. 1981; Hurley et al.1984).

The DR beta chains have one asparagine-linked oligosaccharide at position 19 while the alpha chain has two asparagine-linked amino acids at positions 78 and 116 (Strominger 1980; Shackelford and Strominger 1982). Biochemical studies using tunicamycin to inhibit the formation of asparagine-linked sugars or endoglycosidases to remove sugars have shown that the polymorphism is restricted to the polypeptides with the carbohydrates contributing very little to the heterogeneity (Shackelford et al. 1983). Recent reports by Nepom et al. (1983) however suggest that the Dw variants Dw4 and Dw14 differ only in the post-translational modification of the molecules due to oligosaccharide differences, but this has not been confirmed.

1.1.1B. Polymorphism at the DNA level

There are at least three subregions in the HLA-D region with a minimum of six alpha genes and eight beta genes identified so far. Most of the genes for each subregion were mapped using a combination of cDNA and genomic cloning (reviewed by Korman et al. 1985; Trowsdale et al. 1985). The order of the subregions (Figure 3) is mostly established although the whole region has not yet been mapped with overlapping cosmids (reviewed by Trowsdale et al. 1985). The map (adapted and modified from Bodmer et al. 1984a) is based on studies involving molecular data as well as on recombinations within families (Shaw et al. 1980) and HLA-deletion mutants (Kavathas et al. 1981).

In each of the DP and DQ subregions there are two pairs of beta and alpha genes but only one pair for each subregion is definitely known to be expressed. It is thought that one pair of the DP genes ($\beta 2\ \alpha 2$) is not expressed since both the genes contain frameshift mutations. Recently it has been shown that both the $\beta 1$ gene and the $\alpha 1$ gene are polymorphic. Increased recombination between DP and DQ, possibly due to a hot spot telomeric to DP, is said to explain the lack of linkage disequilibrium between DP and the other class II molecules.

One pair of the DQ genes is highly polymorphic whereas there is limited polymorphism of the other pair which is

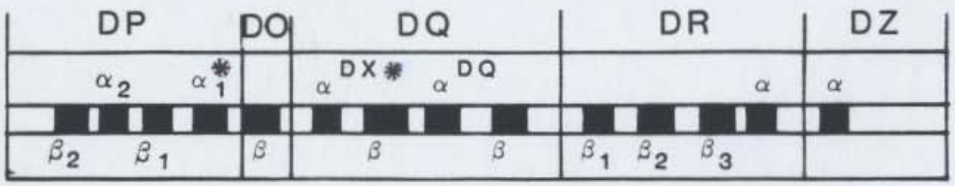

Figure 3. Schematic map of the HLA-D region genes, adapted from Bodmer (1984). The placement of the DO beta gene between DP and DQ is based on the work of Tonnelle et al. (1985) but it may lie between DQ-DR and the complement genes. *putative recombination hot spots.

sometimes called DX (Trowsdale et al. 1985). Recent work at the protein level suggests that some homozygous cells do express two different DQ molecules (Giles and Capra 1985). Both DQ α and β are highly polymorphic and show strong linkage disequilibrium with each other and with the DR beta genes. This was the rationale that Bodmer et al. (1984a) used for placing them together and to the right of two putative recombination hot spots. The other two DQ genes (DX α and DX β) are less polymorphic than the other set of DQ genes. Although they are in linkage disequilibrium with each other, they are not in linkage disequilibrium with either the DQ genes or the DR beta genes. Again this explains why they were placed next to each other and between the two hot spots.

The DR subregion has been placed telomeric to DQ by analogy with the I-A and I-E subregions of the mouse. In addition there is some evidence of a rare crossover that puts DQ centromeric to DR (reported in Giles and Capra 1985). The DR subregion consists of only one alpha gene but the number of beta genes may be as high as four depending on the haplotype. One of the beta genes appears to be a pseudogene (Trowsdale et al. 1985; Moller et al. 1985). Unlike the alpha genes of the DQ subregion, the DR alpha is not polymorphic but the functional beta genes are highly polymorphic with the exception of the DR second beta gene which encodes the DR supertypic specificities, DRw52 and DRw53.

In addition there may be other subregions. An alpha gene originally called DT, then renamed DO (Inoko et al. 1985), appears to be the same as or allelic to the DZ alpha gene (Trowsdale et al. 1985) and has been mapped to the D-region. Recently, a cDNA clone containing a beta gene, called DO was isolated from the mRNA of a hemizygous cell line (Tonnelle et al. 1985). Using mapping techniques, they have shown that it is most likely situated either between the DP genes and the DQ-DR genes or between the DQ-DR genes and the complement genes.

Charron et al. (1985) and Giles and Capra (1985) have reported that an additional source of polymorphism is possible with the expression of hybrid DQ molecules on the cell surface. Apparently the alpha chain produced from the DQ gene of one chromosome can transassociate with the beta chain produced from the DQ beta gene of the other chromosome to produce a new DQ molecule.

1.1.1C Serological definition

Molecules encoded by the HLA-D subregions have proven difficult to define serologically and biochemically using standard typing reagents such as alloantisera and heteroantisera. This is mainly due to the many polyclonal reactivities that are present in antisera. Even so-called monospecific antisera often react with two or more closely related

determinants that are present on different molecules. In addition, most of the alloantisera have low affinity constants, are low titer and are in limited supply.

During the 1984 Histocompatibility Workshop a total of 10 DR specificities (1-w10) were confirmed by serological typing mainly using alloantisera. There was no evidence that DR1, 3, 7, w9 and w10 molecules carried more than one allelic variant. Splits of DR5 (w11 and w12) and DRw6 (w13 and w14) were recognized on the basis of reactions with antisera and monoclonal antibodies (Betuel et al. 1984; Schreuder et al. 1984). Although not given workshop status, DR2 appears to have at least two serological variants (Singal et al. 1985; Mervart et al. 1983), while DR4 appears to have three serological variants (Williamson et al. 1984). DRw8 is quite complicated and will likely be split when specific antisera or monoclonal antibodies become available.

The DR4 specificity has proved especially difficult to characterize with standard typing reagents and it was found to correlate poorly with the cellularly-defined Dw4 specificity (Reinsmoen and Bach 1982). There are several possible explanations for this including the presence of antibodies to the products of closely related genes, such as those encoding the DRw53 and DQw3 specificities in alloantisera which can confuse the results. As previously mentioned DR4 has tentatively been subdivided into three serotypes on the basis

of reactions with three clusters of alloantisera analyzed in the last workshop. In addition several investigators using MLC and immunochemical analyses have shown that DR4 is a supertypic specificity which includes several subtypes. The subtypes defined by MLC (Reinsmoen and Bach 1982; Thompsen et al. 1983) correlate well with the structural variations seen in the DR beta chains that were immunoprecipitated with monoclonal antibodies and analyzed by 2-D gels (Groner et al. 1983; Nepom et al. 1983).

Recent immunochemical analysis of DQ molecules derived from homozygous cell lines showed that these are at least as complex as DR molecules (Giles and Capra 1985). There appear to be three variants of alpha chains and five variants of beta chains so that each DR type is associated with a distinct DQ type. For example, although both DR4 and DR5 are associated with DQw3, the DQ in each type is a different allelic variant. Therefore, it seems likely that the serologically-defined DQ specificities have several subtypes. Furthermore the presence of short antibodies in alloantisera to DQ allelic variants would be almost impossible to distinguish from the closely associated DR specificities. Stastny et al. (1984) have shown that some of the alloantibodies to DR specificities also contain antibodies to specific DQ variants.

1.2 Monoclonal Antibodies to Class II Antigens

From my own experience and judging from the abundance of reports in the literature, the production of murine monoclonal antibodies which recognize monomorphic cell surface determinants is relatively easy. However it is more difficult to make monoclonal antibodies capable of distinguishing the specific product of one gene or allele from that of a closely related gene or allele. Many of the monoclonal antibodies that have been described are subregion specific such as NEI-anti Ia which recognizes monomorphic determinants on DR molecules (Hansen et al. 1980). Others in addition to recognizing all the products of one subregion may also recognize an allelic product of another subregion. For example CA-206, which recognizes a monomorphic DR determinant, has been shown recently to recognize a determinant on DQ molecules of DR7 cells (Charron et al. 1984).

As has been pointed out by Giles and Capra (1985), many of the original monoclonal antibodies were poorly described because the complexity of the D-region was not fully appreciated and many of the cells used to characterize the specificities were not always homozygous. Nevertheless they were, and continue to be invaluable for immunochemical analysis.

Monoclonal antibodies were tested properly for the first

time in the 1984 International Histocompatibility Workshop. Of 110 HLA-D region monoclonal antibodies that were submitted, 92 were accepted for further testing (Bodmer et al. 1984b). In the final analyses, 54 of these were grouped into twenty clusters depending on the product recognized. There were two clusters for DQw1, one each for DQw3, TA10 (subset of DQw3), DRw52, DR4, and the rest of the clusters included more than one known or unknown specificity.

The third listing of the monoclonal antibody registry (Colombani et al.1984) included all those submitted to the workshop as well as previous entries (Colombani et al. 1982; Colombani et al. 1983) and new entries up to June, 1984. Of the class II monoclonal antibodies, 35 (44.9%) were "defined polymorphic" while 10 (12.8%) were "undefined polymorphic", 12 (15.4%) were "defined monomorphic" and 21 (26.9%) were "undefined monomorphic". Only a minority of the polymorphic antibodies recognized the classical antigens defined by alloantisera and these were: one each for DR1-like, DR2-activated cells, DR3 + monomorphic DQ, DR4, DR5, DR7, DR7+w15, DRw8+w15, DRw6+w8, DR1+4+10, DR1+4-like, DR2+4+6, DR7+3+5+6; two each for DR4+1+9 and DRw53; three for DRw52. The remaining monoclonal antibodies recognized polymorphisms defined as either DQw1, DQw3, or related specificities.

When one considers that 19 HLA-D region variants were serologically recognized in the Ninth International Workshop

(Albert and Mayr 1985), and that numerous laboratories have been engaged in the production of monoclonal antibodies to HLA antigens for almost a decade, it is surprising that there is still a paucity of HLA-D region monoclonal antibodies for routine HLA-D typing reagents. However, it must be pointed out that in addition to those discussed above, there are other reported monoclonal antibodies to polymorphic determinants, but either they are not cytotoxic (all antibodies were tested by complement-dependent cytoxicity in the last workshop) or have been described since the workshop.

Although it is disappointing that most of the monoclonal antibodies have not proved useful for serological analysis, they have been invaluable, particularly in immunochemical analyses, in increasing our understanding of class II molecules. Because monoclonal antibodies are ultraspecific reagents, they are often capable of discriminating between two closely related determinants. For example in the last workshop, DRw13 (a DRw6 split) could be distinguished from DRw12 (a DR5 split) on the basis of their reactions with four monoclonal antibodies (Schreuder et al. 1984). Crepaldi et al. (1985) has described an interesting broadly reacting monoclonal antibody which reacts with DR1, DRw8, and DRw9 but can apparently differentiate between DRw13 and DRw14 (DRw6 subtypes) and between Dw10 and Dw4 (DR4 subtypes). Hence even a monoclonal antibody with a broad specificity can be a

useful typing reagent.

1.3 Preparation of Monoclonal Antibodies to HLA-D Region Molecules

With the advent of monoclonal antibody technology (Kohler and Milstein 1975), several investigators have attempted to produce monoclonal antibodies which recognize specific determinants on HLA molecules. Since there are several good review articles (Goding 1980) and textbooks (Kennett et al. 1981; Goding 1983) which describe in detail the preparation of monoclonal antibodies, only a brief overview of the technique as it pertains to this work will be reviewed here. Emphasis will be placed on assays used to screen for and characterize monoclonal antibodies to HLA antigens.

1.3.1 Technique

1.3.1A Immunization

While some investigators (Brodsky et al. 1979) have used semi-purified HLA molecules prepared from cell lysates, others have simply use whole cells to immunize mice, usually Balb/c (Trucco et al. 1979). The dose and the number of immunizations are variable but generally two immunizations

several weeks apart are given intraperitoneally and a final immunization given intravenously three days prior to fusion. In most cases the animal will respond well to cellular antigens but the response is predominately species-specific. Since finding a specific B-cell making antibody to a particular HLA antigen among the numerous other immune B-cells in the spleen is technically difficult most investigators perform somatic-cell fusions without previous selection.

1.1.3B. Fusion procedure

There are several 8-azaguanine resistant myeloma lines that are deficient in the enzyme, hypoxanthine-guanine-phosphoribosyltransferase (HGPRTase) which make suitable fusion partners. It is preferable to use one of the non-secretors such as SP2/0-Ag14 (Shulman et al. 1978).

Fusion techniques vary from one laboratory to another with respect to the composition and concentration of the polyethylene glycol, the ratio of spleen cells to myeloma cells, the duration of the hybridization, the culture medium, supplements, and the use of a feeder layer. Hybrids are selected in HAT medium, which contains thymidine and hypoxanthine as the exogenous sources of DNA nucleotide precursors for the salvage pathway (Littlefield 1964). They are required because the <u>de novo</u> pathway is blocked by the aminopterin, a folic acid analog, which stops the HGPRTase deficient myeloma

cells from growing. Only fused cells which express the HGPRTase enzyme from the spleen cell partner will proliferate. The unfused spleen cells are incapable of continuous growth in vitro and die naturally. A fusion and subsequent cloning of positive hybrids can result in hundreds of microcultures that require an efficient screening method.

1.3.2 Screening for HLA-specificities

1.3.2A Complement dependent cytoxicity

For approximately twenty years, HLA serologists have used the complement-dependent cytoxicity (CDC) assay (Terasaki and McClelland 1964) to define the HLA antigenic system and to characterize alloantisera which are used to HLA type and crossmatch patients and their prospective organ donors. Reactions of many of the monoclonal antibodies are not detectable by CDC. Either their Fc regions do not bind the complement component, C1q, efficiently as is the case for IgG1 antibodies or they bind epitopes too far apart to be cross-linked by C1q (Trucco et al. 1980). This crosslinking is required for the initiation of the classical complement cascade. Such technical problems are rarely encountered when antisera with their many polyclonal specificities are used. In order not to miss monoclonal antibodies against rare specificities, it is important therefore to use an alternative method that is

sensitive, specific, and reproducible. It should, also be simple and fast because successful fusion and cloning can yield hundreds of cultures that require immediate screening since new hybrids are unstable (Goding 1980).

1.3.2B Cell binding assays

Many of the standard immunological assays including indirect immunofluroscence and immunoperoxidase staining are tedious to perform on large numbers of samples and have the disadvantage of reader bias. Solid phase assays such as radioimmune assays (RIA) and enzyme-linked immunosorbent assays (ELISA) are usually preferred. Most of the original RIA were performed on cells in suspension and specific monoclonal antibody that bound to the cells was detected with ^{125}Iodine-labelled rabbit or goat anti-mouse immunoglobulins. Certain drawbacks including the inherent danger of using ^{125}I, its relatively short half-life, and the numerous centrifugation steps required, led several investigators to develop the ELISA as an alternative method.

Although the RIA as described by Tsu et al. (1980) was originally used to detect NFLD.M1, it was decided to evaluate an ELISA that used cells stuck to poly-L-lysine treated wells of a microtitre plate followed by fixation with glutaraldehyde (Stocker and Heusser 1979; Kennett 1981). Despite the obvious advantages of having cells adhered to plastic, this

method had to be abandoned because the glutaraldehyde selectively destroyed some HLA-DR epitopes and increased non-specific binding. The method that was found to be the most reliable was the cellular enzyme-linked immunosorbent assay (CELISA) performed essentially as described by Morris et al. (1982). In principle it is similar to the ELISA but viable untreated cells are used instead of glutaraldehyde-fixed cells. This method was used for all the screening and specificity testing and is described in detail in Chapter II.

1.4 Objectives

One of the monoclonal antibodies (NFLD.M1) produced in this laboratory appeared to react with a polymorphic determinant on human B-lymphocytes. The main objectives of this project were:

1) to determine the specificity of NFLD.M1 using a cellular enzyme-linked immunoassay (CELISA)

2) to study the expression of the M1 epitope on different cells.

3) to do preliminary molecular analysis of the molecules immunoprecipitated by M1.

CHAPTER II MATERIALS AND METHODS

2.1 Production of NFLD.M1 Monoclonal Antibody

2.1.1 Immunization protocol

1×10^7 B-lymphocytes from a chronic lymphocytic leukemic patient with the HLA phenotype: A2,A11; B15,Bw35; Cw3, Cw4; DR4, were injected intraperitoneally (IP) into a female neonatal mouse, less than 24 hours old. This was followed by further injections at 1 week, 2 weeks and 6 weeks. A final immunization was given at age 3 months and the fusion was done 3 days later.

2.1.2 Fusion procedure

2.1.2A Reagents

i. Hybridoma medium (HM)

500 ml RPMI 1640 (Flow Laboratories General, McLean, Virginia 22102 USA)

100 ml heat-inactivated fetal bovine serum (FBS), (Flow Laboratories)

10 ml 200mM L-glutamine (Flow Laboratories)

5 ml 5×10^{-3}M 2-mercaptoethanol (2-ME), (Sigma Chemical

Co. St Louis, Missouri 63178 USA).

ii. 100 x HAT solution

136 mg hypoxanthine (Sigma), 1.76 mg aminopterin (Sigma) and 38.8 mg thymidine (Sigma) were dissolved in 80 ml dH_2O with a few drops of 5M NaOH added to facilitate dissolving. It was then made up to 100 ml, sterilized through 0.22 µm filter (Flow Laboratories) and stored at -20°C in 5 ml aliquots.

iii. 100 x HT solution

This was prepared in the same way as 100 x HAT, but the aminopterin was omitted.

iv. HAT medium and HT medium

One ml of either 100 x HAT or 100 x HT was added to 100 ml HM to give a final concentration of 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine.

v. Polyethylene glycol (PEG), 50%

Two gm. PEG mw.4000 (J.T.Baker Co., Phillipsburg, N.J.), was melted by autoclaving. Two ml prewarmed serum free RPMI-1640 (SF-RPMI) was added to the PEG when it had cooled to approximately 50°C.

2.1.2B Fusion partner

The mouse myeloma line, SP2/0-Ag14 (Shulman et al. 1978) was a gift from Dr. Robert Weaver, Vancouver. It does not secrete immunoglobulin chains and is deficient in the enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPRTase) and is therefore resistant to 8-azaguanine.

The cells were grown in RPMI-1640 containing 10% FBS, 2mM L-glutamine, and 1 mM sodium pyruvate (Flow Laboratories), and maintained in midlog phase (3×10^5) with a viability greater than 90%. Revertants were discouraged by growing in medium containing 8-azaguanine, 30 ug/ml, (Sigma) every 10 passages.

SP2/0 cells were counted using a hemacytometer and the viability was determined using phase contrast microscopy, or alternatively by Trypan blue exclusion as follows:

> 0.1 ml well-mixed cells + 0.9 ml 0.3% Trypan blue (3 ml 1.0% stock solution and 7.0 ml phosphate buffered saline-PBS) were mixed. A hemacytometer was filled and the number of live (unstained) and dead (stained blue) cells in the complete area, (0.9 mm^2) were counted. Viable cells per ml were calculated by multiplying live cells counted x dilution x 10000/9.

2.1.2C Spleen cells

The immunized mouse was anesthetized with ether and killed by cervical dislocation. The spleen was removed using

aseptic technique and placed in a petri dish which contained 5-10 ml SF-RPMI. The cells were teased from the organ using a pair of sterile hypodermic needles bent at 90° and mounted on syringe barrels, and resuspended in SF-RPMI. The clumps were allowed to settle and the cell-rich supernatant was collected in a 15 ml conical tube (Falcon, Oxnard, Cal. USA). The cells were washed twice in SF-RPMI and counted in a hemacytometer.

2.1.2D Fusion

The fusion was done essentially as described by Oi and Herzenberg (1980). The appropriate number of myeloma cells (1 myeloma:10 spleen cells) were centrifuged in a 50 ml conical tube (Falcon) at 500g/10 min and washed twice in SF-RPMI. They were then combined with the spleen cells, washed twice with SF-RPMI, decanted and all the supernatant was removed by inverting the tube on sterile gauze and allowing it to drain.

One ml prewarmed PEG was slowly stirred into the pellet over 2 min, followed by the addition of 2 ml of prewarmed SF-RPMI stirred in over 2-4 minutes. An additional 8 ml medium was then added over 5-8 min. The cell mixture, which was quite clumpy, was centrifuged at 500g/10 min and the supernatant was discarded without disturbing the button. Ten ml medium was vigorously added and the cells were centrifuged again.

The cells were resuspended very gently in HM at a concentration of 10 x 10^6/ml and dispensed in 0.1ml aliquots

to 96-well microtitre trays (master plates). At the same time control cells (SP2/0 and spleen cells) were plated at the same density in each of two rows.

2.1.3 Selection and maintenance of hybrids

2.1.3A Selection

The plates were incubated at 37°C in an incubator containing 10% CO_2 and left for 24 hours before 2 x HAT was added. Feeding was done by removal of roughly one half the medium and addition of fresh HAT medium on days 3, 5, 8, 11, and 14. Hybrids were visible microscopically on day 7 and macroscopically on day 14, so the medium was changed to HT. When cell growth was approximately 50% confluent (day 14 to day 21), the supernatants were screened against the immunizing cell.

2.1.3B Transfer and Cloning

As soon as a positive hybrid was identified by a suitable screening assay (Section 2.2), it was transferred to a 24 well plate (Linbro, Flow Laboratories) which contained cloning medium (CM), (HT medium plus 2 x 10^7 RBC/ml and 5 x 10^6 spleen cells/ml from an unimmunized Balb/c mouse). After 1 wk the hybrids in the transfer plate were cloned by limiting dilution (CLD) essentially as described by Oi and Herzenberg (1980).

The cells were counted as described above, diluted to 50 cells/ml in 4.6 ml CM, and plated at 0.1 ml/well (5 cells) in the first three rows of a microtitre tray. Five ml CM was added to the 1.0 ml that remained, mixed and plated at 0.1 ml/well (1 cell) in the next three rows. Then 1.2 ml CM was added to the remaining 1.2 ml and plated at 0.1/ml (0.5 cell) in the last two rows.

The cells were fed on day 5 and every third day until 50% confluency was achieved. The supernatants were tested for specific antibody using immunizing cells and an appropriate panel of cells. Positive clones were selected from rows in which the percentage of wells with growth, according to the Poisson distribution, indicated that the cells were derived from a clone (Goding 1980). These were expanded for freezing and further testing.

2.1.3C Expansion

The cells in the selected well were gently mixed with a Pasteur pipette and transferred to a well in a 24-well plate that contained 1 ml CM. Feeding was done every three days until confluent growth was achieved (approximately 10 to 14 days). The cells were then transferred to a 50 ml flask (Falcon) containing 5 ml HM and fed 1:2 twice weekly until there was 50 ml at a density of 5×10^5/ml with a viability greater than 90%. Most of the cells were frozen at this stage and the supernatants were retested to ensure that the cells

were still secreting antibody. In addition some cells were expanded and overgrown to achieve maximum antibody production.

2.1.3D Freezing

The cells were counted and for every tube to be frozen (Nunc tube, Gibco Laboratories, Grand Island, NY, USA), 2 x 10^6 cells were removed. The cells were centrifuged at 500 g/10 min. The supernatant was saved for antibody testing and the cells placed on ice. The cells were resuspended at 2 x 10^6/ml in cold freezing solution which is 10% dimethylsulphoxide (DMSO), (J.T.Baker Chem. Co.) and 90% FCS, and dispensed in 1 ml aliquots per prechilled Nunc tube. The cells were frozen in a programmed cell freezer (Cryo-Med Model 700, Mt. Clemens, Michigan, USA) at the rate of 1^o/min and then placed in liquid Nitrogen (LN_2).

2.1.3E Thawing

Cells were thawed quickly by agitating at 37^oC until only a crystal of ice remained, pipetted into a tube containing 10 ml HM and washed twice to remove DMSO. Cell counts were done and if the viability was good (greater than 60%), they were resuspended at 2 x 10^5/ml HM in a 50 ml flask. If it was less than 60%, the cells were plated in a 24 well plate with a feeder layer, as described for cloning.

2.1.4 Purification of MFLD.M1

2.1.4A Ascites fluid

For each clone two Balb/c mice were prepared by injecting 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, Wisconsin 53201 USA) IP twice, 10 days apart. Three days after the last pristane injection 2 x 10^6 hybridoma cells were given IP. When the ascites fluid developed (10-14 days later), the fluid was harvested as follows. The abdominal skin was sterilized with 70% alcohol and a butterfly needle, gauge 21, was inserted and adjusted until fluid flowed down the tube. This was collected aseptically, and the monoclonal antibody-rich fluid was separated by centrifugation (500g/10 min.) and stored at -20°C.

i. Ammonium sulphate precipation

The ascites fluid was diluted 1/2 with 0.15M NaCl and enough saturated ammonium sulphate (SAS), (J.T. Baker Chem. Co.) was added to give 45% SAS. This mixture was stirred at RT/30 min and centrifuged at 1000g/15 min/4°C and the precipitate was washed twice with 40% SAS. The final precipitate was dissolved in the minimum amount of PBS. Ammonium sulphate ions were removed using a Sephadex G10, PD-10 column (Pharmacia) which had been pre-equilibrated with PBS.

ii. Protein-A Affinity Chromatography

NFLD.MI was purified from the SAS precipitated protein solution on a Protein A Sepharose 6M (Pharmacia) column which was prepared according to the manufacturer's instructions. The sample, diluted with Tris-buffered saline, pH 8.6, was added and buffers ranging from pH 8.6 to pH 2.5 were used to elute the proteins (Ey et al. 1978).

2.2 Screening Assays

NFLD.M1 was identified using a solid phase radioimmune assay (RIA), (Tsu and Herzenberg 1980), but due to technical problems discussed in Chapter IV, it was decided to switch to an enzyme-linked immunosorbent assay (ELISA) for screening NFLD.M1 clones and characterizing the antibody. Considerable effort was spent in adapting an ELISA on glutaraldehyde-fixed (GA-F) cells (Kennett 1981) but this assay unfortunately gave both false positives and false negatives. Although it was abandoned in favor of a live cell ELISA, CELISA (Morris et al. 1982), it produced significant findings which should be of interest to all those involved in producing monoclonal antibodies (MCAB) to cell-surface antigens (Drover and Marshall, submitted).

2.2.1 Radio Immune Assay (RIA)

This assay was performed essentially as described by Tsu and Herzenberg (1980). Briefly, 20 ul target cells (2×10^7/ml) in phosphate buffered saline (PBS) containing 5% FBS and 0.02% Sodium azide (RIA buffer) were mixed with 20 ul MCAB supernatant in U-bottom wells of a PVC plate (Dynatech Laboratories, Inc., Alexandria, Virginia, 22314 USA). After 1 hr incubation at 4°C the cells were washed 3x with RIA buffer by centrifuging the plates at 500g/10 min at 10°C and aspirating the wash solution. ^{125}I-RAM was prepared by labelling $F(ab')_2$ fragment rabbit anti-mouse IgG (heavy + light chains) (Cappel Laboratories, Cochranville, PA., 19330 USA) with sodium ^{125}Iodine (Amersham Corp., Arlington Heights, IL.,60005 USA). Fifty ul (40,000 CPM) of this ^{125}I-RAM was added/well. After incubation for 1 hr at 4°C the cells were washed 4 times. The plates were dried, the wells cut apart and the radioactivity counted in a gamma counter.

2.2.2 Enzyme-linked immunosorbent assays

2.2.2A Reagents used for both ELISA-GAF and CELISA

i. <u>1.5M phosphate-buffered saline (PBS) 10X</u>

80 gm NaCl

2 gm KH_2PO_4

21.7 gm $Na_2HPO_4 \cdot 7H_2O$

2 gm KCl

Dissolved in 1 liter distilled H_2O and diluted 1/10 for working solution.

ii. Poly-L-Lysine (PLL) 1 mg%

1mg PLL-hydrobromide, molecular weight 180,000 (Sigma) dissolved in 100 ml PBS and stored at 4°C.

iii. Blocking Buffer (BB)

90 ml PBS-BSA + 10 ml normal goat serum (Gibco, Grand Island, NY 14072 USA) + 0.3 gm gelatin (Sigma).

iv. Control Antibodies

a) NEI-011 anti-human Ia (New England Nuclear, Boston, MS 02118 USA), subclass IgG2a, (Hansen et al. 1980).

b) CA-206, a monomorphic DR MCAB, subclass IgG2b, (Charron and McDevitt 1979).

c) AT.AB-anti HLA-DR, subclass IgG1, (Atlantic Antibodies, Scarborough, Maine 04074-0060 USA).

d) OKT-3, a pan-T cell MCAB, subclass IgG2a) and OKT-8, anti-T8, IgG2a) (Ortho, Raritan, New Jersey, USA)

e) Reference mouse myeloma proteins (Litton Bionetics, Inc., Kensington, Md. 20795 USA) included the following: TEPC-183 (IgMk), MOPC-21 (IgG1k), UPC-10 (1gG2ak), MOPC-195 (IgG2bk), Y5-606 (IgG3λ), and MOPC-315 (IgAλ_2).

v. Conjugate

Peroxidase-labelled affinity purified goat anti-mouse immunoglobulins (heavy and light chains), (Kirkegaard and Perry, Richmond, Cal 94804), were diluted in PBS containing 2% BSA.

vi. Phosphate Citrate Buffer

a) 0.1M citric acid (J.T. Baker Chemical Co.) - 19.2 gm in 1000 ml dH_2O.

b) 0.2M phosphate - 28.2 gm Na_2HPO_4 (J.T. Baker Chemical Co.) in 1000 ml dH_2O.

12.2 ml (a) plus 12.7 ml (b) were mixed with 50 ml dH_2O and the pH adjusted to 5.0 with (a) or (b).

vii. Substrate

4 mg of chromogen, orthophenylenediamine dihydrochloride (OPD), (Sigma) dissolved in 10 ml PCB.

4 ul 30% H_2O_2 (J.T. Baker Chemical Co.) added, immediately prior to use.

2.2.2B ELISA on GA-fixed cells

i. Cell Coated Plates: Target cells were washed in PBS at 500g/10 min, counted and adjusted to 1 x 10^7/ml. Fifty ul PLL was added per well of a PVC-U bottom microtitre plate (Dynatech) and incubated at RT/30min. PLL was removed by

flicking and 50 µl (5×10^4) cells was added per well. The plates were centrifuged at 300g/10 min in microtitre carriers (Cooke). Fifty µl cold glutaraldehyde (GA), (Sigma), 0.5% in PBS was added to each well and incubated at RT/15 min. GA was removed by flicking and the plates were washed twice in PBS by immersion and flicking. The wells were filled with glycine buffer, 100 mM glycine (Sigma) in PBS containing 0.1% BSA, left at RT/30 min and washed twice in PBS. Two hundred µl 0.1% BSA was added per well and the plates were either used immediately or stored at -20°C.

ii. Assay: All washes in the following procedure were done by immersion of the plates in 0.5% Tween-80 (Sigma) in PBS (PBS-T), then flicking and tapping vigorously to remove the wash solution. The wells were filled with BB and incubated for 1 hr/RT to decrease non-specific binding and washed twice. Ten or 25 µl MCAB, appropriately diluted in 10% FCS in RPMI, was added to each well and incubated at RT or 37°C/hr followed by three washes. Fifty µl conjugate was added/well and incubated at 37°C/hr. The cells were washed 4x in PBS-T followed by one wash in PBS. One hundred µl substrate was added/well and incubated at 37°C/30 min. The reaction was stopped by adding 50 µl 2.5N H_2SO_4/well and the absorbance read at 492 nm, using a Multiskan Spectrophotometer (Flow Laboratories).

2.2.2C. ELISA on live cells (CELISA)

This assay was performed essentially as described by Morris et al. (1982). Briefly, target cells were washed twice in PBS and resuspended at 5×10^6/ml in PBS-BSA. Ten µl cells was mixed with 10 µl antibody in V bottom wells of PCV plates (Dynatech). After 1 hr at RT the cells were washed 3x in PBS-BSA by centrifuging the plates at 500g/5 min at 10°C and flicking to remove the wash solution. Fifty µl conjugate, optimally diluted (1/800) in 2% PBS-BSA, was added/well and incubated 2 hr. The cells were washed 4x and the plate was snap-fitted onto an EIA plate (Flow Laboratories) which had been precoated with PLL. One hundred µl PBS was added/well, the cells were resuspended and holes were punched in the center of each well with a 16 gauge needle. The plates were centrifuged at 500g/10 min so that cells passed into the lower wells and adhered to the PLL-coated wells. Fluid was removed by flicking. Colour was developed as described for ELISA-GA-fixed cells.

2.3 Target Cells

2.3.1 Cell lines

Lymphoblastoid cell lines were mainly used as target cells. The class, HLA phenotype and source are given in

Appendix A. All cells were grown in RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine, and 1mM sodium pyruvate (Flow Laboratories). Cells were harvested for assays when they were in logarithmic phase and were greater than 90% viable.

2.3.2 Isolation of T- and B-lymphocytes

2.3.2A Separation of peripheral blood mononuclear cells

Mononuclear cells were separated from heparinized blood using Ficoll Hypaque (FH) density gradient centrifugation (Boyum 1968). Briefly, blood was diluted 1/2 in PBS and layered over 10 ml FH (Pharmacia density = 1.077) in a 50 ml conical tube (Falcon). The layers were centrifuged at 500g/20 min and the mononuclear cells at the FH-plasma interface were collected in RPMI-1640. The cells were washed 3x at 500g/10 min. They were resuspended in warm RPMI-1640, containing 10% FBS and held at 37°C.

2.3.2B Monocyte depletion

Monocytes which have endogenous peroxidase were depleted from the PBM suspension using Sephadex G10 (Pharmacia), (Chien & Ashman 1984). A 10 ml-disposable syringe was plugged with glass wool and packed with 5 ml Sephadex G10. The column was washed with 10 ml warm RPMI-1640 containing 10% FBS and the warm PBM cells (1×10^7) were layered on top of the column. When the medium flowed through, the column was closed

and left at 37°C for 30 min. The nonadherent cells were eluted with warm medium.

2.3.2C Separation of T- and B-lymphocytes

These were separated by a combination of E-rosetting and removal of rosettes by FH (Weiner et al. 1978). Non-adherent cells were counted and adjusted to 5 x 10^6/ml and 2.5 ml was added to 2.5 ml neuraminidase-treated sheep erythrocytes, mixed for 15 min, and centrifuged at 300g/5 min. The rosettes were gently resuspended and separated by FH as described above. The B-lymphocytes were collected at the FH interface and T-lymphocytes were obtained from the pellet using 0.87% ammonium chloride to disrupt the erythrocytes.

2.3.3 PHA-Stimulated T-Cells

T-cells, obtained as described above, were cultured in RPMI-1640 medium containing 10% FBS, 2mM L-glutamine and phytohemagglutinin (PHA) 1/80 (Gibco) at 1 x 10^6/ml. The cells were grown at 37°C with 10% CO_2 for 72 hr at which time transformation was evident macroscopically (large aggregates of cells) and microscopically.

2.4 Isotyping and Quantification of NFLD.M1

2.4.1 Ouchterlony analysis

Double diffusion in 1% agarose was performed by a standard method (Ouchterlony) using the following rabbit anti-mouse immunoglobulins (Litton Bionetics): IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and anti-lambda and anti-kappa light chains. Myeloma proteins listed in section 2.2.2A were used as positive controls.

2.4.2 Quantification using ELISA

2.4.2A Reagents

In addition to those listed in section 2.2.2.A the following were also required:

i. Bicarbonate buffer pH 9.6

1.59 gm Na_2CO_3 (J. T. Baker Chemical Co.)

2.93 gm $NaHCO_3$ (J. T. Baker Chemical Co.)

Dissolved in 1 l dH_2O and stored at RT for not more than 2 weeks.

ii. Goat anti-mouse (GAM) IgG1

GAM-IgG1 (Litton Bionetics) diluted 1/500 in bicarbonate buffer for coating plates.

2.4.2B Assay

Two hundred/ul GAM antibodies was added to each well of an EIA titration plate, incubated overnight at 4°C and flicked out. Two-hundred ul BB was added/well and left 1 hr at RT and the plates were washed once in PBS-T. Two-hundred ul MCAB or myeloma protein-standards appropriately diluted in PBS-T was added to replicate wells and incubated 1 hr at RT. The wells were washed 4x in PBS-T and 200 ul conjugate was added /well and incubated at RT for 1 hr. After 4 washes in PBS-T 200 ul substrate was added and color developed at RT in the dark for 30 min. The reaction was stopped with 50 ul 2.5N H_2SO_4/well and read in the Multiskan spectrophotometer.

The concentrations of the standards were plotted versus adjusted optical density (OD) (average of replicate samples) on semilog paper and the MCAB concentration was calculated.

2.5 Molecular Weight Determination of NFLD.M1 Determinant

2.5.1 Iodination of cell surface proteins

The cells were counted, centrifuged and washed 3x in PBS. Four x 10^7 cells in 200 ul PBS was added to a glass tube coated with 100 ug Iodogen, (Pierce Chemical Co., Rockford, Il 61105), (Markwell and Fox 1978). One mCi ^{125}Iodine, specific activity 15.3mCi/mmol, (Amersham @ Searle) was added

and the mixture incubated on ice/10 min with occasional mixing. The cells were washed 5x in cold PBS at 500g/5 min and lysed in 1 ml lysis buffer, 0.5% NP-40 (Sigma) in PBS containing 1mM phenylmethylsulfonylfluoride (Sigma), for 30 min as described by Shackelford et al. (1981).

The lysate was transferred to a 1 ml Eppendorf tube, centrifuged in a microfuge ((Eppendorf) at 15000g/15 min. and the supernatant, containing the crude membrane fraction, was collected. It was either used immediately or stored at -70°C.

2.5.2 Immunoprecipitation

One hundred ul lysate was reacted with 5-10 ug MCAB/1 hr in an Eppendorf tube. Ten ug RAM-IgG1 was added because mouse IgG1 binds poorly to Protein A (Goding 1978) and incubated 1 hr/RT. One-hundred ul 25% PA-Sepharose, which had been washed extensively in NET buffer, was added and incubated at 4°C, rotating overnight. The mixtures were washed 5x in NET buffer. The immune complexes were eluted from PA-Sepharose with 50 ul 2x Laemmli sample buffer (Laemmli 1970) and heated at 100°C/5 min.

2.5.3 One-Dimensional Electrophoresis (SDS-PAGE)

2.5.3A Reagents

i. 30% Acrylamide +0.8% BIS

30 gm acrylamide (Bio-Rad), Richmond, Cal. 94804 USA) and 0.8 gm N'N'methylene-bis-acrylamide (BIS), (Bio Rad) dissolve in 100 ml dH_2O. Filtered and stored at 4°C.

ii. Lower Tris (4x) 1.5M Tris-HCl, pH 8.8 + 0.4% SDS

18.17 gm Trizma base (Sigma) + 4 ml 10% sodium dodecyl sulphate (SDS), (Bio-Rad) in 80 ml dH_2O; adjusted pH with 12N HCl and made up to 100 ml.

iii. Upper Tris (4x) 0.5M Tris HCl, pH 6.6 + 0.4% SDS

6.06 gm Trizma base + 4 ml 10% SDS in 80 ml dH_2O; adjusted pH as above and made up to 100 ml.

iv. Tris Glycine Resevoir Buffer (4x)

12 gm Trizma + 57.6 gm glycine (Sigma) in 1 l dH_2O.
For running buffer dilute 500ml in 2 l 0.1% SDS.

v. 2% Ammonium pepsulfate (AP)

10 mg in 5 ml dH_2O; prepared fresh.

vi. Sample Buffer

10 ml glycerol (BDH)

+ 5 ml 2-Mercaptoethanol

+ 30 ml 10% SDS

+ 12.5 ml upper Tris

+ 52.5 ml dH_2O

2.5.3B Preparation of gels

i. Lower gel(10%)

11.3 ml dH_2O

7.0 ml lower tris (4x)

9.3 ml 30% acrylamide + 0.8% BIS

0.007 ml N,N,N',N'-tetramethylethylenediamine (TEMED), (Bio-Rad)

0.4 ml 2% AP

The first 4 reagents were mixed, degassed, the AP added and poured immediately. The gel was overlayed with a few drops of isopropanol and allowed to polymerize for at least 2 hr.

ii. <u>Upper Gel or Stacking Gel</u>

3.17 ml H_2O

+ 1.25 ml upper tris (4x)

+0.5 ml 30% acrylamide + 0.8% BIS

+ 0.005 ml TEMED

Mixed, degassed and 0.075 ml 2% AP added.

The isopropanol was removed from the lower gel which was then rinsed with 1x lower tris and blotted dry. The comb

was inserted and the stacking gel was pipetted on top of the lower gel and polymerized for 1 hr.

2.5.3C Electrophoresis

The clamps and cams were removed from the plates, which were then placed in a tank containing 1600 ml running buffer. 400 ml running buffer was added to upper reservoir, the combs removed and the wells rinsed with buffer. 30 ul sample, prepared as described above (2.5.2), was loaded per well and electrophoresed at 50 V (15mA) until samples entered the running gel. The voltage was then increased to 150 V (50mA) and electrophoresis continued until the Brom-phenol blue in each sample was 1 cm from the bottom of the gel.

2.5.3D Autoradiography

After electrophoresis the gel was dried for 2 hr using a gel dryer (Bio-Rad) and then autoradiographed on a Kodak X-Ray film with an intensifying screen for two days at -70°C.

2.6 Analysis of CELISA Data

2.6.1 Specificity testing

Duplicate testing was done for each cell type with NFLD.M1, a positive control, usually NEI-Ia (a monomorphic DR monoclonal antibody) and a negative control, usually a

non-specific IgG1 myeloma protein or culture medium. The OD for each pair was averaged and the background OD was subtracted to give an adjusted OD (Adj. OD) value. If the OD of each duplicate varied from the mean by more than 15%, or if the OD of the positive control was less than 3 times the background, the test was rejected. The reactivity of NFLD.M1 was related to NEI-Ia by expressing the Adj.OD of NFLD.M1 as a percentage of the Adj. OD for NEI-Ia (% NEI-Ia).

The CELISA results for NFLD.M1 were designated either positive or negative according to their distribution in a Frequency Distribution plot which was done using the Adj. OD values for NFLD.M1, NEI-Ia and the % NEI-Ia. Two by two contingency tables were set up in order to do a correlation analysis between NFLD.M1 and each DR or DQ specificity. The significance of each correlation coefficient (r value) was calculated using chi square and Fisher's exact test. The methods are as follows:

i. The correlation coefficient:

$$r = \frac{ad-bc}{\sqrt{(a+b)(c+d)(a+c)(b+d)}}$$

ii. The chi square method:

a) $X^2 = \frac{N([ad-bc]-N/2)^2}{(a+b)(c+d)(a+c)(b+d)}$ (with Yates' correction)

b) $p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{N!\,a!\,b!\,c!\,d!}$ (Fisher's exact test)

where a,b,c,d are cells in the appropriate 2 x 2 contingency table for NFLD.M1 and the antigen being compared, p is the probability and N is the total number of cells.

2.6.2 Comparison of NFLD.M1A to NFLD.M1B

To find if two sources of NFLD.M1 (A = purified antibody, 5 ug/ml, from ascites fluid produced with G3 clone; B = supernatant from the overgrown G3-H11 subclone) reacted in a similar manner, both were reacted in CELISA with several cell lines. The results were compared using Spearman's rank order method of doing a correlation coefficient when the distribution is not normal and the significance of the correlation coefficient (p) was tested using the Student's t test. The methods used were as follows:

$p = 1 - \frac{6\Sigma(D^2)}{N(N^2-1)}$ where D = the difference between rankings
N = the number of results

$$t = \frac{p\sqrt{N-2}}{\sqrt{1-p^2}}$$

where p = Spearman's correlation coefficient

N = number of pairs

CHAPTER III RESULTS

3.1 Preliminary Testing of Uncloned Culture

The fusion from which NFLD.M1 was derived produced over 100 hybrids. Unfortunately, all except nineteen were lost due to contamination by yeast. Supernatant from the master cultures and culture medium as a negative control were tested for antibody activity using the immunizing cells (NB) in a radioimmune assay (RIA). Specific antibody was detected using I^{125}-labelled GAM (40,000 CPM/well), so that the amount of Iodine (CPM) bound to the cells could be directly related to the amount of specific antibody bound to the cell surface. The ratio of the average test CPM:average background CPM (cells + culture medium) was calculated for each cell. Ratios greater than 2.5 were considered positive. All except for one were negative (data not shown).

Supernatant from this hybrid (NFLD.M1) was then tested against several lymphoblastoid cell lines (LCL) including NB in an RIA (Figure 4). The supernatant reacted strongly with the immunizing cell and with GM3190, to a lesser degree with GM3162 and GM3160 but did not react with the remaining LCL including the T-cell line, GM2219 (Figure 4). Since the supernatant contained antibody activity which apparently

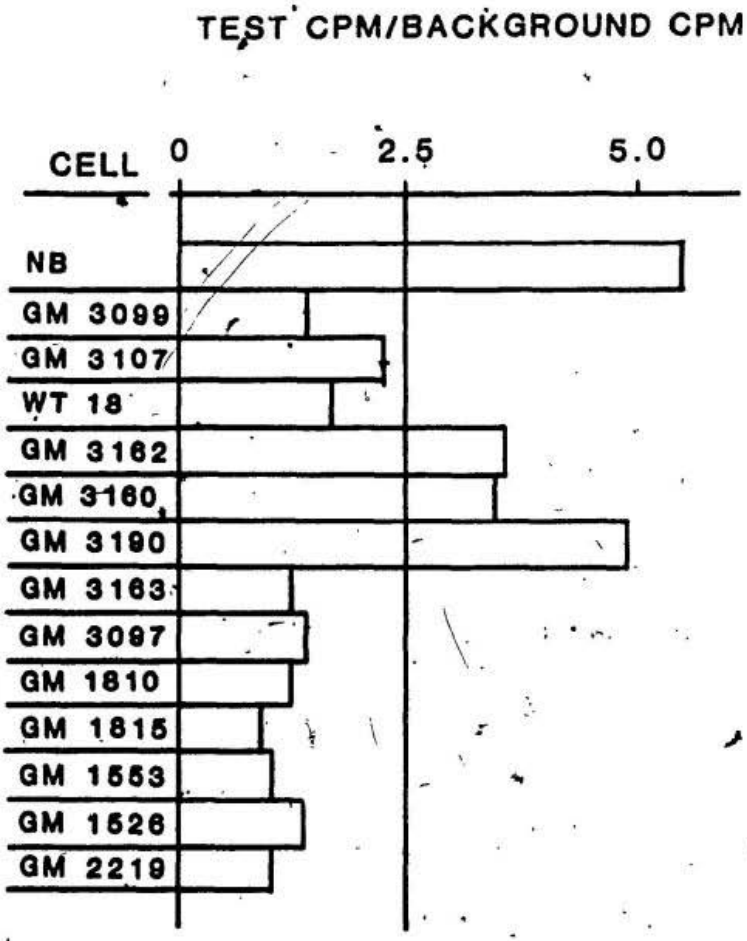

Figure 4. Preliminary screening of NFLO.M1 using RIA. Supernatant from the master culture was tested against the immunizing cell (NB) and a panel of cell lines. Ratios greater than 2.5 were considered positive.

recognised a polymorphic determinant on the cell surface, the master culture was expanded, the supernatant collected, and the cells cloned.

3.2 Selection of a Screening Assay

After the preliminary screening with RIA, it was decided to adapt the enzyme-linked immunoassay (ELISA) using poly-L-lysine to stick cells to wells of a microtiter plate, followed by glutaraldehyde (GA) fixation as described in Chapter II. This assay gave confusing and conflicting results (data not shown). To find an explanation for these results experiments were performed to test the specificity of the assay. These are described in the following sections.

3.2.1 ELISA on GA-Fixed Cells gave non-specific results

An experiment designed to test the specificity of the assay involved reacting GA-fixed cells (GM3161) with various concentrations of mouse myeloma proteins which had been procured as standards for immunochemistry. Three of the proteins reacted non-specifically with the cells (Figure 5). The most nonspecific binding occurred with IgM, which was still strongly reactive at 3.125 µg/ml, whereas IgG2a and IgG2b adhered only at higher concentrations (>10 µg/ml).

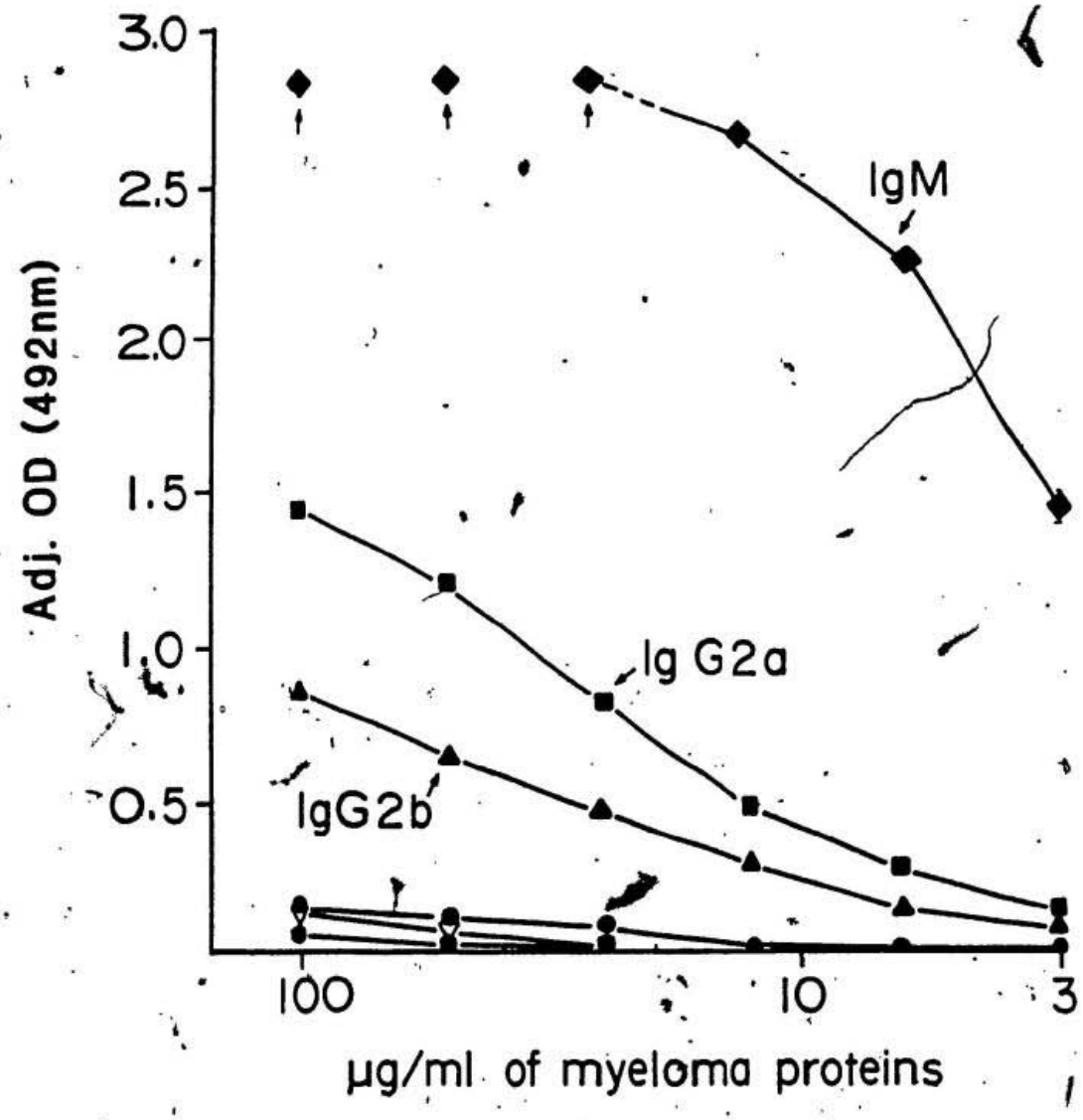

Figure 5. Reactions of irrelevant myeloma proteins in ELISA using GA-fixed cells (GM 3161). The OD values were adjusted (Adj. OD) by subtracting the background (culture medium) OD from the test OD. IgM (◆) > 10 µg/ml produced OD values too high to read; IgG2a (■) and IgG2b (▲) reacted non-specifically with the cells, whereas IgG1 (●), IgG3 (▽) and IgA (●) were non-reactive.

There was little binding of IgA, IgG1 or IgG3 even at high concentrations; nor was there any significant binding of any myeloma proteins to plastic alone, (data not shown). These findings showed that ELISA results could easily be interpreted as positive when hybridomas were secreting high levels of non-specific antibody, particularly when the antibody is IgM.

3.2.2 Comparison of ELISA on GA-fixed cells versus CELISA

The next experiment was designed to compare binding of two non-specific myeloma proteins, IgM and IgG2b, with two specific monoclonal antibodies (MCAB), NEI-anti-IA (IgG2b) and ATAB-DR (IgG1) using GA-fixed cells and viable cells. The viable cell assay (CELISA) was performed essentially as described by Morris et al. 1981. Both assays were carried out simultaneously on GM3190 cells.

3.2.2A Variability within replicates

The OD value for each test was adjusted (Adj. OD) by subtracting the OD of the background (culture medium). The average Adj. OD for each duplicate was calculated and the variability of each method was evaluated. This was done by calculating the percent variation (%V) of each duplicate from the mean Adj. OD. The mean (X) and standard deviation (s) of the %V values for each assay were then calculated. As can be

seen in Table 2 which shows a sample of the data, the agreement between duplicates was generally good for both methods, but the variability was greater with live cells than with fixed cells.

3.2.2B Non-specific binding

As can be seen from Figure 6 the data confirmed the previous finding that IgM and IgG2b reacted non-specifically with GA-fixed cells. In addition they also reacted non-specifically with viable cells, but to a lesser degree. Even at the lowest concentration, 1.562 µg/ml, IgM reacted 5 times more with GA-fixed cells than with the viable cells whereas IgG2b binding approached background levels at 10 µg/ml. Neither IgM nor IgG2b bound non-specifically to the poly-L-lysine and glutaraldehyde-treated plastic.

3.2.2C Glutaraldehyde modifies some D-region molecules

Testing with the specific MCAB on viable and GA-Fixed cells (Figure 7) showed that NEI anti-Ia reacted poorly with the fixed cells even at 10 µg/ml and at 2.5 µg/ml it was completely non-reactive. The binding that did occur at 10 µg/ml was probably non-specific since it occurred in the range in which the irrelevant IgG2b bound non-specifically to GA-fixed cells (Figure 6). Binding of NEI anti-Ia to viable cells (Figure 7) was dramatically increased compared to that of the fixed cells. At 0.313 µg/ml binding was still above

Table 2. Reactions expressed as Adj. OD* of viable cells and glutaraldehyde fixed cells (GA-F) in ELISA using two monoclonal antibodies.

	NEI-Ia[a]				IgG2b[a]			
	Viable cells		GA-F cells		Viable cells		GA-F cell	
ug/ml	OD*	%V	OD	%V	OD	%V	OD	%V
100	ND	ND	ND	ND	775	10.2	1146	3.9
50	ND	ND	ND	ND	516	9.4	778	1.8
25	ND	ND	ND	ND	303	13.1	526	3.7
12.5	ND	ND	ND	ND	121	7.6	304	3.4
10.0	800	4.2	ND	ND	ND	ND	ND	ND
5.00	853	21.7	143	2.6	93	2.5	190	6.0
3.13	ND	ND	ND	ND	3	0.6	99	4.4
2.5	840	4.3	56	4.0	ND	ND	ND	ND
1.56	ND	ND	ND	ND	0	1.1	52	0
1.25	598	3.3	54	1.0	ND	ND	ND	ND
0.63	523	12.9	37	2.9	ND	ND	ND	ND
0.31	228	22.2	35	5.4	ND	ND	ND	ND
0.16	113	1.2	14	4.5	ND	ND	ND	ND

Table 2. continued

	NEI-Ia		IgG2b[a]	
	Viable cells %V	GA-F cells %V	Viable cells %V	GA-F cells %V
X	10.0	3.4	7.2	3.9
s	9.0	1.6	4.8	1.4

OD* = Adj. OD x 10^3; X = mean of %V; s = standard deviation.

a. IgG2b is an irrelevant MCAB whereas NEI-Ia is a specific MCAB.

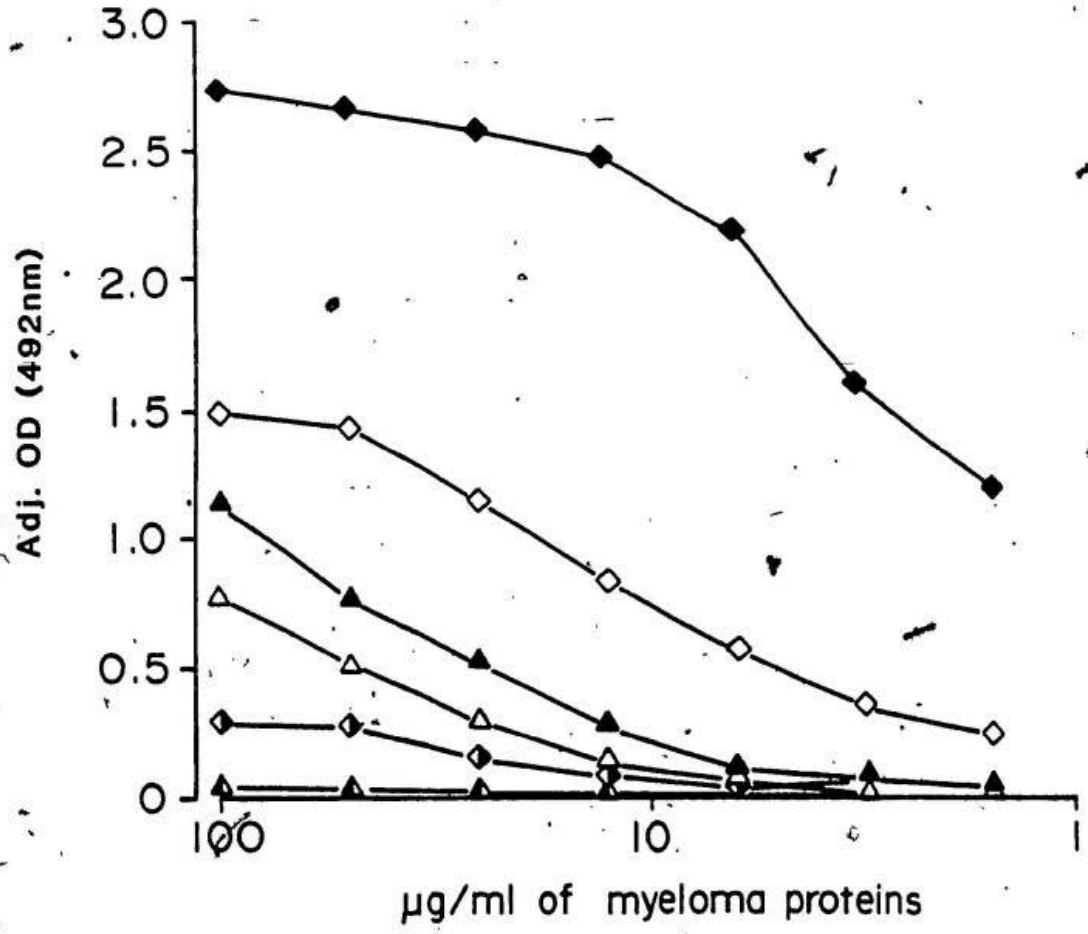

Figure 6. Non-specific binding of two irrelevant myeloma proteins to GM3190 cells in ELISA. IgM (diamonds) and IgG2b (triangles) were reacted with GA-fixed cells (closed symbols), viable cells (open symbols) and poly-L-lysine GA-treated plastic (half-filled symbols).

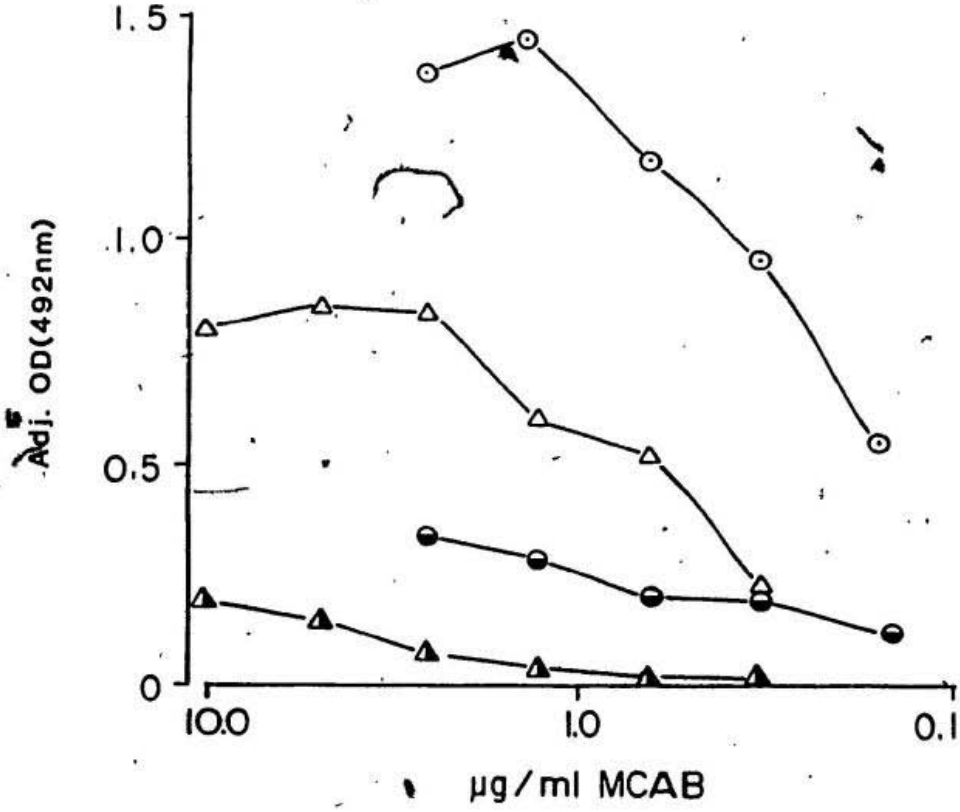

Figure 7. Binding of two monomorphic DR MCAB to GM3190 cells as measured in ELISA: NEI-Ia (triangles) and ATAB-DR (circles) on GA-fixed cells (half-filled symbols) and viable cells (open symbols).

background levels.

ATAB-DR reacted with both viable and fixed cells (Figure 7). It is expected that the binding to GA-fixed cells was specific, since it was an IgG1 and this subclass did not bind non-specifically to GA-fixed cells (Figure 5). The reaction with the viable cells, however, was considerably stronger than that with the GA-fixed cells. This suggested that the antigenic determinant was at least partly destroyed or altered in contrast to the NEI anti-Ia determinant which appeared to be completely altered by GA-fixation.

3.2.3 CELISA selected for screening assay

It was obvious from the preceding data that the risks of selecting false positives from hybridoma cultures were greater using GA-fixed cells, particularly if the antibody was IgM or if the immunoglobulin concentration was too high. Even more alarming was the fact that some HLA-DR antigens, and possibly other cell surface antigens, were denatured to such an extent that specific antibodies no longer recognized them. Although the ELISA using GA-fixed cells was simpler and less time consuming than using viable cells, its lack of specificity and sensitivity compared to CELISA eliminated it as a screening method.

3.2.3A Optimal conditions for CELISA

Before implementing the CELISA as a routine screening method, optimal conditions for the conjugate concentration and antigen density were established.

i. Cross-titration of conjugate: To find the optimal dilution of the conjugate, a cross-titration of ATAB-DR against three dilutions of GAM-HRP (1/400, 1/800, and1/1000) was done using constant antigen density (1 x 10^5 GM3190 cells/well). NEI anti-IA 2.5 ug/ml and culture medium were also included in each assay. The background OD values 0.129, 0.112, and 0.104 respectively were acceptable for all three conjugate dilutions. The duplicates in the GAM-HRP 1/400 titration were variable but there was excellent agreement between the duplicates in the other titrations (Figure 8). In addition, the OD values for ATAB-DR (>2.5 ug/ml) with 1/400 conjugate were too high to be read accurately by the Multiskan. When NEI anti-Ia (2.5 ug/ml) was expressed as a percentage of ATAB-DR (2.5 ug/ml) there was very little difference in the values obtained, 37.6%, 34.9%, and 38.85% respectively, for all three dilutions of conjugate. However, GAM-HRP 1/800 seemed to be optimal in the ATAB-DR titration so that dilution was chosen for future testing.

ii. Antigen concentration: The next experiment was designed to establish optimal cell density since Morris et al. (1981)

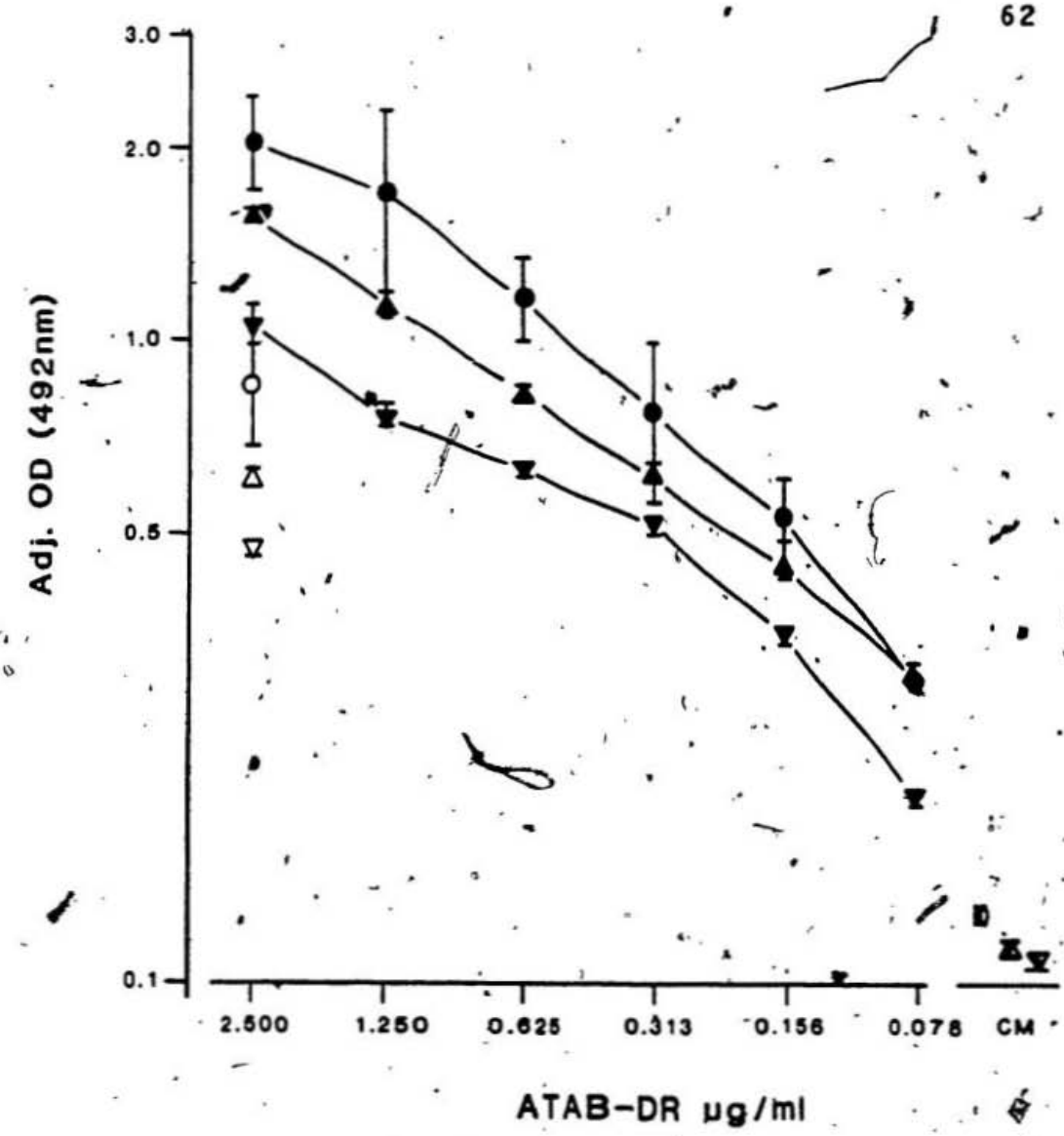

Figure 8. Effect of diluting the conjugate on measuring the reaction of ATAB-DR in CELISA. Three dilutions of GAM-HRP, ●—● 1/400, ▲—▲ 1/800, ▼—▼ 1/1000 were cross-titrated against doubling dilutions of ATAB-DR, in CELLSA; range and mean of OD values are indicated for each point. NEI-Ia 2.5 µg/ml (open symbols) and culture medium (half-filled symbols) were included as positive and negative controls, respectively.

had reported that antigen excess could depress specific antibody binding. Four concentrations of cells (GM3161) were tested with NEI anti-Ia (2.5 ug/ml) and culture medium. There was little evidence of depressed antibody binding due to high antigen concentrations (Figure 9), but below 2.5×10^4 the sensitivity decreased. Since there was very little difference in sensitivity at concentrations between 5×10^4 and 20×10^4, and since it was sometimes necessary to economize on cells, 5×10^4/well was chosen for the antigen concentration.

3.3 Preliminary Screening of NFLD.M1 in CELISA

Before cloning NFLD.M1, the stored supernatant and two commercial MCAB were tested in CELISA against cells from 4 CLL patients (Table 3A), 5 B-LCL and 1 T-LCL (Table 3B). The two positive controls reacted appropriately. ATAB-DR was positive with all cells except GM2219 (Molt-4), which is a T-cell line and does not express DR molecules while ATAB-ABC reacted with all cells except GM3190 (Daudi) which does not express HLA-ACB. NFLD.M1 antibody reacted with some of the B-cells but not with GM2219. The data suggested that NFLD.M1 recognized a polymorphic determinant on B-cells, possibly a DR polymorphism.

Since the number of DR molecules present on the cell surface has been shown to vary with the different stages in

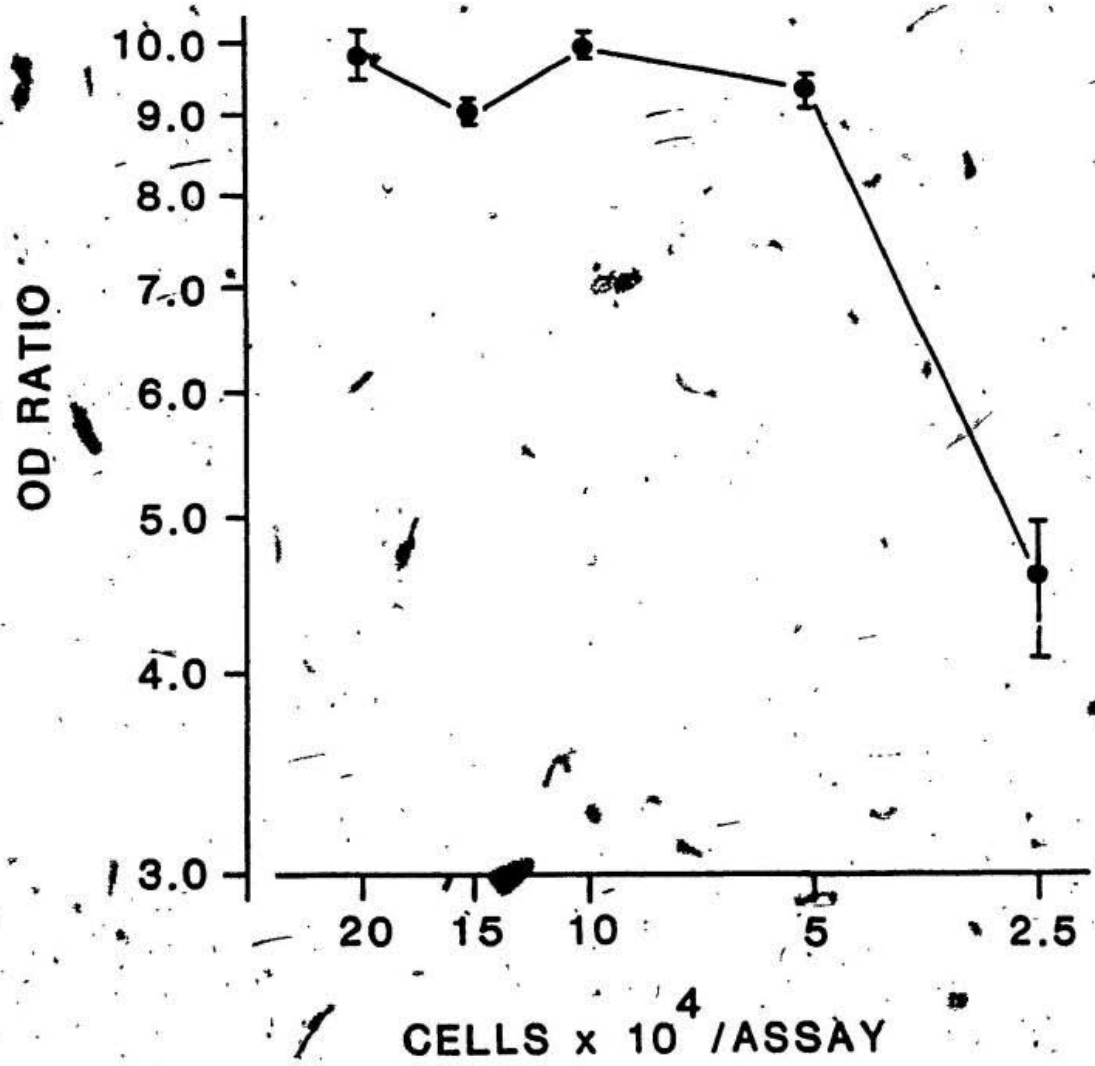

Figure . Effect of cell concentration on measuring the reaction of NEI-Ia and culture medium with GM 3190 cells in CELISA. Five concentrations of cells were reacted with NEI-Ia, 2.5 µg/ml. The results were expressed as an OD ratio (Test OD/Background OD) with the range and mean indicated.

Table 3A. HLA phenotypes and CELISA results of CLL-cells

	CLL			
	FS4115	FS3425	FS5059	FS3029
HLA:A	2,28	2,25	3,	1,2
C	3,5	N	4	N
B	44,40	7,18	7,35	8,14
DR	4	3	1,7	2,3
MCAB:		Adj. OD*		
ATAB-DR	1238	1121	1283	472
ATAB-ABC	1141	963	1115	868
NFLD.M1	473	3	13	7

*Adj. OD x 10^3

Table 3B. HLA phenotypes and CELISA results of LCL

	LCL					
	GM3104	GM3161	GM3105	GM3190	GM3163	GM2219
HLA:A	3,3	3,3	26,28	N0	30,30	1,10
C	4,4	2,2	N	N	6,6	?
B	35,35	7,7	38,18	N	13,13	?
DR	1,1	2,2	5,5	5,6	7,7	N
MCAB:	Adj. OD*					
ATAB-DR	1306	983	1636	1558	490	20
ATAB-ABC	940	344	1167	112	933	470
NFLD.M1	230	178	107	407	48	6

*Adj. OD x 10^3 N = not detected

the cell cycle (Sarkar et al. 1980), it seemed more informative to express the NFLD.M1 reactivity as a percent of the ATAB-DR reactivity (Figure 10). This was done as follows:

$$\frac{\text{OD NFLD.M1} - \text{OD Background}}{\text{OD ATAB-DR} - \text{OD Background}} \times 100\%$$

It was interesting that the only CLL cell to react was FS4115 which shared HLA: A2 and DR4 with the immunizing cells. However, two of the nonreactive CLL cells, also were typed HLA-A2 which suggested that A2 was not the NFLD.M1 determinant. Since there was some reaction with at least two of the B-LCL cells, it was postulated that the antibody was directed to a DR determinant. There was no point in any further interpretation of the data since the supernatant was not obtained from a cloned culture.

3.4 Cloning

Cloning by limiting dilution was efficient with 100% growth in the first 36 wells (5 cells/well), 86% in the second 36 wells (1 cell/well), and 21% in the last 24 wells (0.5 cell/well). Supernatants were collected three days after the last feeding and approximately half were selected on the basis of cell growth and tested for specific antibody

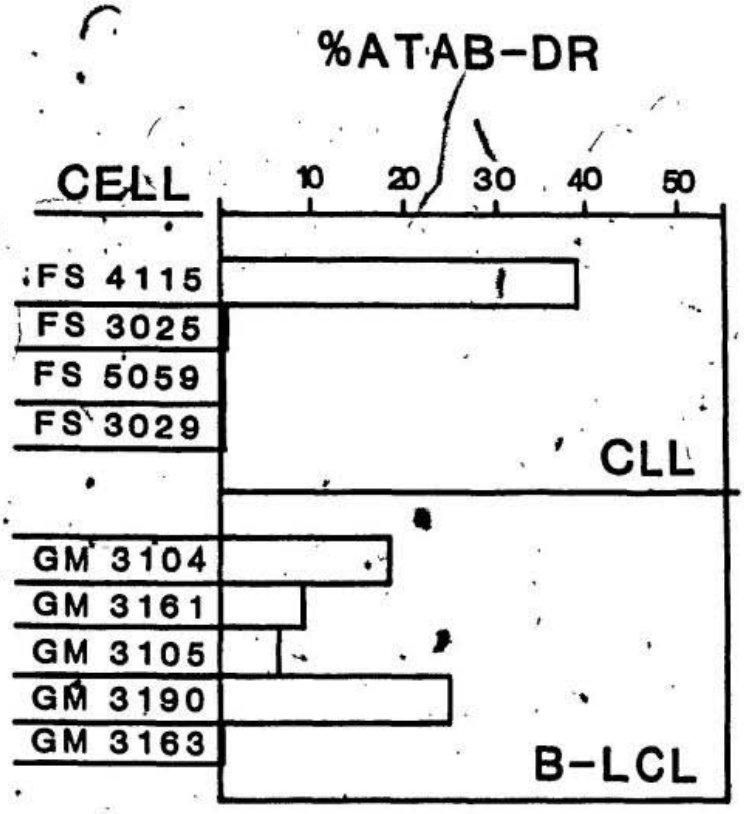

Figure 10. Reactions of NFLD.M1 obtained by screening the supernatant from the master culture in CELISA against a panel of CLL and B-LCL. The results of each test are given as %ATAB-DR reactivity which is the Adj. OD values for NFLD.M1 expressed as a percentage of the Adj. OD values for ATAB-DR, 1/800).

Nine of the cultures were selected, expanded and frozen for further study. Supernatants, collected from confluent cultures, were tested against 6 cells including the last aliquot of immunizing cells (Table 5). Three cultures (D4, E2, and E9) were discarded because they were either weakly positive or negative. Although A1, A6, C7 and E6 reacted more strongly with the target cells, they were not selected for characterization because each was less likely to be derived from a single cell than were G3 and H4. The latter two had been cloned at 0.5 cell/well and since there was only 21% growth in the wells cloned at 0.5 cell/well, they were most likely derived from a single cell according to the Poisson distribution.

Both G3 and H4 were injected IP in pristane-primed Balb/c mice to produce ascites but H4 failed to do so. Ascites fluid collected from the G3 mouse was centrifuged to separate the antibody rich fluid from the cells. The fluid was stored at -70°C and the cells were cloned.

3.4.1 Subcloning

Using the ascites cells from the G3 mouse, 100% cloning efficiency was achieved in the first 36 wells (5 cells/well), 47% in the second 36 wells (1 cell/well), and 17% in the final 24 wells (0.5 cell/well). Twelve supernatants from the

Table 4. Results of cloning by limiting dilution.

Group[a]	# wells with growth	# wells tested	# SN positive*/LCL			
			GM3190	GM3105	GM3161	GM2219
I	36	18	18/18	6/18	13/15	0/15
II	31	18	14/18	0/15	5/15	0/15
III	5	5	2/2	0/2	1/5	0/2

a. Group I was plated at 5 cells/well; Group II, at 1 cell/well and Group III, at 0.5 cells/well.

* Supernatants (SN) from some of the clones were tested in CELISA on some of the cells shown above. OD values > 3x background were considered positive.

Table 5. CELISA results as Adj. OD* on supernatant from selected clones (C) tested against various cells.

	CELLS					
C	NB[a]	GM3190	GM3105	GM3104	GM3161	FS5059
A1	1553	633	411	303	151	43
A6	1559	734	704	NT	NT	NT
C7	1384	737	574	NT	NT	NT
D4	47	8	2	3	0	25
E2	491	233	61	7	38	24
E6	1263	716	506	146	74	21
E9	436	361	369	78	37	18
G3	1088	821	704	380	210	59
H4	1076	561	297	258	140	621

*Adj. OD values x 10^{-3} a = Immunizing cell

wells cloned at 1 cell/well and four supernatants from the wells cloned at 0.5 cell/well were screened for specific antibody using 1 T-LCL (GM2219) 1 myeloid line (K562) and 4 B-LCL. ATAB-DR (1.25 ug/ml) and ATAB-ABC, a class I monomorphic MCAB (1.25 ug/ml) were included as positive controls.

As can be seen in Table 6 all supernatants were negative with T-cell and myeloid lines. Fifteen supernatants reacted with GM1488, GM1905 and GM3190 and produced intermediate reactions with GM3160. These cells type HLA: DR4,5; DR4,w6; DRw6 and DRw12 respectively. Three clones (G9, H7 and H11), were selected from the wells cloned at 0.5% of which 17% grew and therefore were most likely derived from a single cell. Prior to freezing the cells the supernatants were rechecked for antibody secretion in order to choose one for characterization. Five B-LCL were tested and the results (Figure 11) showed that supernatants from all 3 clones were reacting with all the cells in a similar way. One clone H11 designated NFLD.M1B was overgrown for antibody production, the supernatant was collected and stored at 4°C.

3.5 Immunoglobulin Isotyping and Quantification.

NFLD.M1 was isotyped IgG1 kappa using supernatant from the overgrown G3-H11 subclone and a set of antisera and appropriate controls in a standard Ouchterlony technique. The

Table 6. CELISA results as Adj. OD* on supernatant from NFLD.M1 Subclones (Sc) tested against various cells.

Sc	B-LCL GM3161	T-LCL GM2219	Myel K562	B-LCL GM3160	B-LCL GM3190	B-LCL GM1905	B-LCL GM1488
D8	48	57	62	230	746	736	792
D10	47	53	28	221	692	748	869
D11	48	61	44	220	796	986	822
D12	43	47	33	164	550	685	781
E1	46	61	47	175	646	528	773
E5	45	53	28	150	701	595	922
E7	43	64	35	164	630	704	770
E8	37	56	35	164	629	747	818
E12	46	55	81	87	135	118	156
F2	40	59	72	288	657	826	1075
F6	47	60	54	295	752	870	1013
F10	39	54	58	293	729	767	1118
G6	38	64	55	157	485	379	570
G9	36	57	43	225	546	528	707
H7	50	63	49	290	580	684	805
H11	40	56	48	202	374	840	879
-DR	57	49	44	1311	1811	1621	2018
-ABC	347	361	255	405	212	1031	1188

Adj.OD* $x10^3$; -DR = ATAB-DR,1/800; -ABC = ATAB-ABC, 1/400

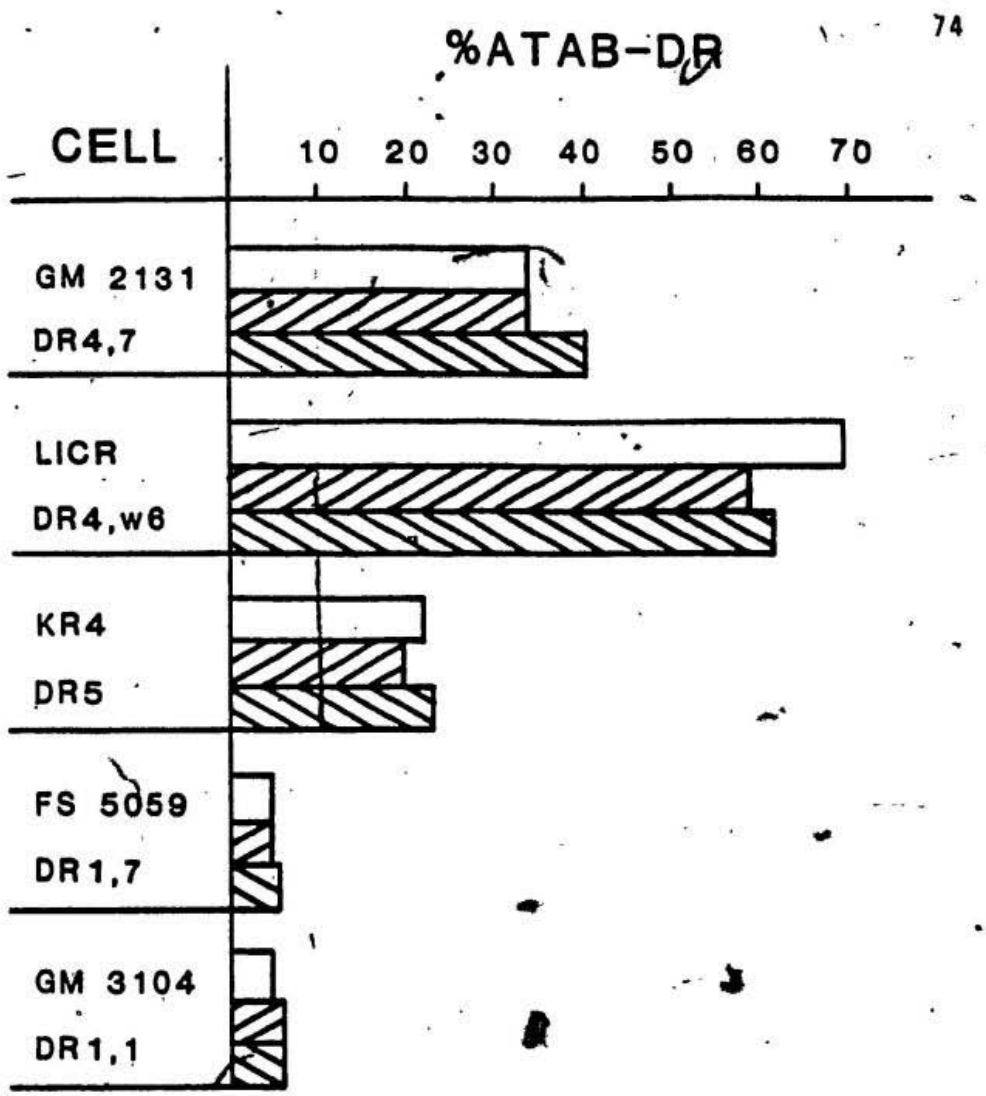

Figure 11. CELISA data on three supernatants from NFLD.M1 subclones expressed as a percentage of the ATAB-DR control. (G9 □ , H7 ▧ , and H11 ▨).

ELISA technique was used to determine the concentration of NFLD.M1 by comparison with a standard curve prepared from IgG1 standards (Figure 12). The NFLD.M1 supernatant, diluted 1/5000, produced an Adj.OD of 0.917 and was calculated to to contain 57.5 ug/ml.

The ascites fluid (G3 clone) was fractionated on a Protein A column as described in Section 2.1.4A. The monoclonal antibody eluted from the column at pH 7.2 which is the pH at which IgG1 no longer binds to Protein A (Ey et al. 1978). The fractions were pooled and the protein concentration was determined by measuring the Absorbance at 280nm. The formula, O.D. x (Dilution factor) x 0.69 mg/ml (Hudson and Hay 1980) was used to calculate the protein concentration (250 ng/ml). The specific antibody was shown to be confined to this fraction with very little found in the excluded fraction. Figure 13 shows the reactions obtained by titrating the Protein-A fraction (NFLD.M1A), the excluded fraction, the supernatant (NFLD.M1B) and NEI-Ia in CELISA using GM3190 cells. The IgG1 concentration for NFLD.M1B was determined by ELISA and the IgG2b concentration for NEI-Ia was given in the packaging enclosure. The results for both sources of NFLD.M1 are almost superimposable and appear be reaching a plateau at 5 ug/ml.

3.60 Serological Pattern on Peripheral Blood Lymphocytes

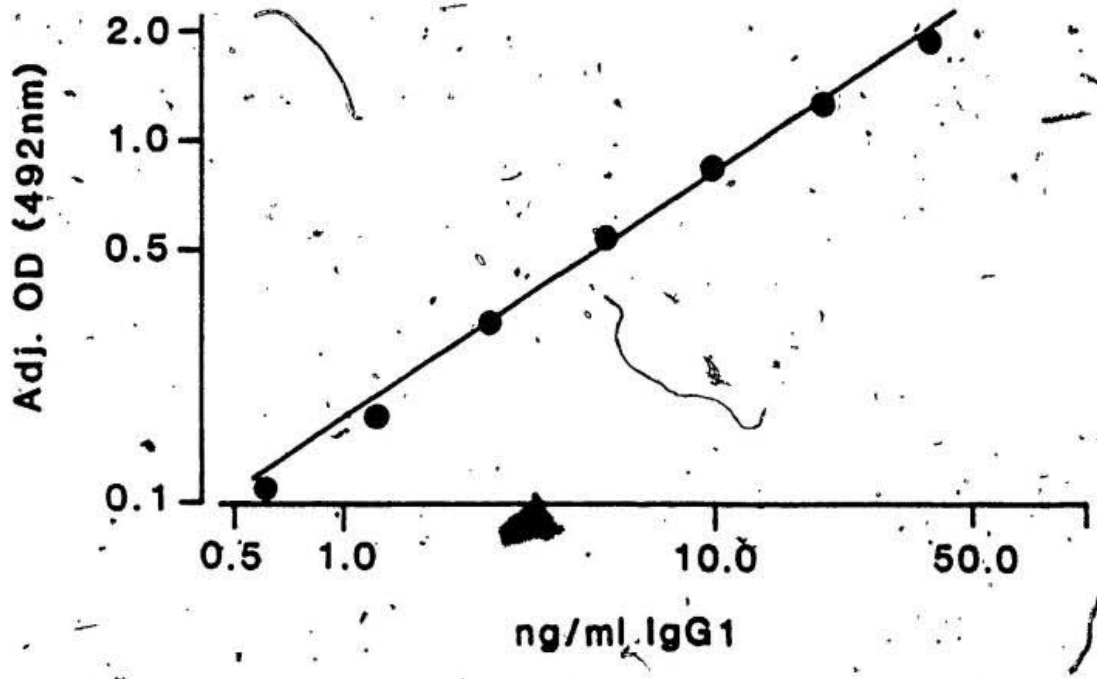

Figure 12. OD as a function of IgG1 concentration measured in ELISA. Mouse IgG1 standards were used to prepare the graph.

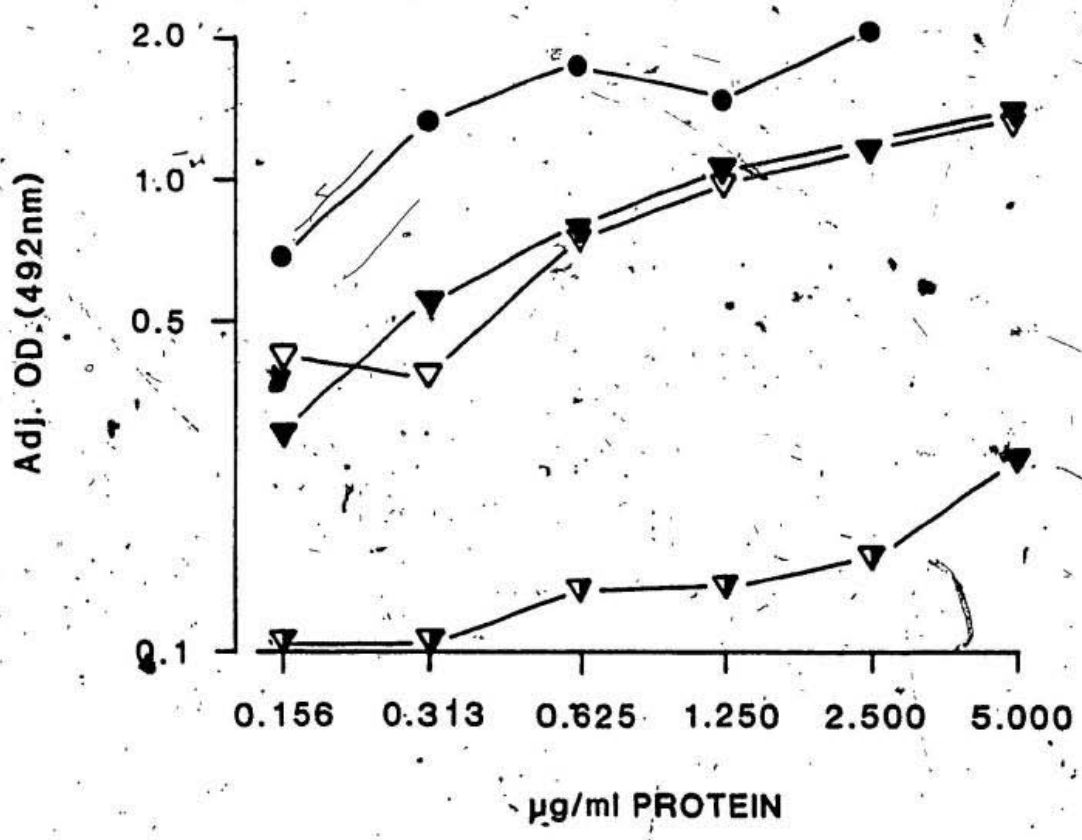

Figure 13. CELISA results obtained for titrations of NFLD.M1 against GM 3190: supernatant (▼), purified ascites fraction (▽), and the excluded fraction (▼). NEI-Ia (●) was included for comparison.

The reactivity of NFLD.MI antibody was studied using CELISA on peripheral blood T- and B-lymphocytes from unrelated healthy donors and some family members. All the individuals tested had been HLA-typed with the Ninth International Histocompatibility Workshop panel of typing reagents.

3.6.1 PBL from unrelated controls

Heparinized blood from 9 volunteers (Table 7A) was separated into T- and B-lymphocytes. Both T- and B-cells were tested at 5 x 10^4/well using NFLD.M1B supernatant, SP2/0-Ag14 supernatant as a negative control, and two positive controls: ATAB-DR which reacts with B-lymphocytes and OKT-8 which reacts with T-lymphocytes. The B-lymphocytes were also tested against 4 dilutions of unpurified NFLD.MIA ascites fluid and an irrelevant ascites fluid, which was procured from a Balb/c mouse that had been given Freund's adjuvant IP. In addition T-lymphocytes were stimulated with PHA, and the PHA-blasts were tested using the same protocol as was used for the resting T-lymphocytes.

3.6.1A Quality of the data

The purity of each B- and T-lymphocyte population was investigated by testing appropriate control MCAB with each population in CELISA (Table 7B and Figure 14). When the Adj.OD value for each OKT-8 + B-lymphocyte combination was

Table 7A. HLA Phenotypes of PBL donors

	PBL donors								
	EE	PH	SB	LSM	ES	SD	HD	BL	WHM
HLA:	Antigens Present								
A	24,30	1	24,31	2,24	2,25	2	2,3	1,23	1,32
C	7	3,6	5	1,3	5	0	3	4	7,8
B	7,27	62,57	8,44	22,27	39,44	5,44	62,60	44,52	8,14
DR	4	4,7	3,4	1,5	11,13	5,7	6,8	2,7	3,7
DRw	53	53	52,53	53	52,53	52,53	52,53	52	52,53
DQ	2	2,3	2,3	1,3	1,3	2,3	1,3	1,2	2

Table 7B. Results as Adj. OD* of MCAB reacted with separated PBL in CELISA

	PBL donor								
Test[a]	EE	PH	SB	LSM	ES	SD	HD	BL	WHM
DR+B	558	1152	723	971	831	827	1009	776	1209
DR+T	86	116	83	91	164	104	186	107	155
DR+P	313	435	254	673	404	403	525	616	557
T8+B	37	-67	6	1	36	24	41	43	61
T8+T	580	963	749	621	1041	760	771	630	650
T8+P	1230	1427	1144	1416	1526	1584	1426	1365	849

Table 7B. continued

Test[a]	PBL donor EE	PH	SB	LSM	ES	SD	HD	BL	WHM
M1+B	330	417	210	77	59	85	151	37	38
M1+T	43	41	14	22	10	16	7	24	19
M1+P	114	101	161	24	9	1	1	2	26

a DR = ATAB-DR,1/800; T8 = OKT-8,1/100; M1 = NFLD.M1B

B = B-lymphocytes; T = T-lymphocytes; P = PHA-stimulated

*OD x 10^3

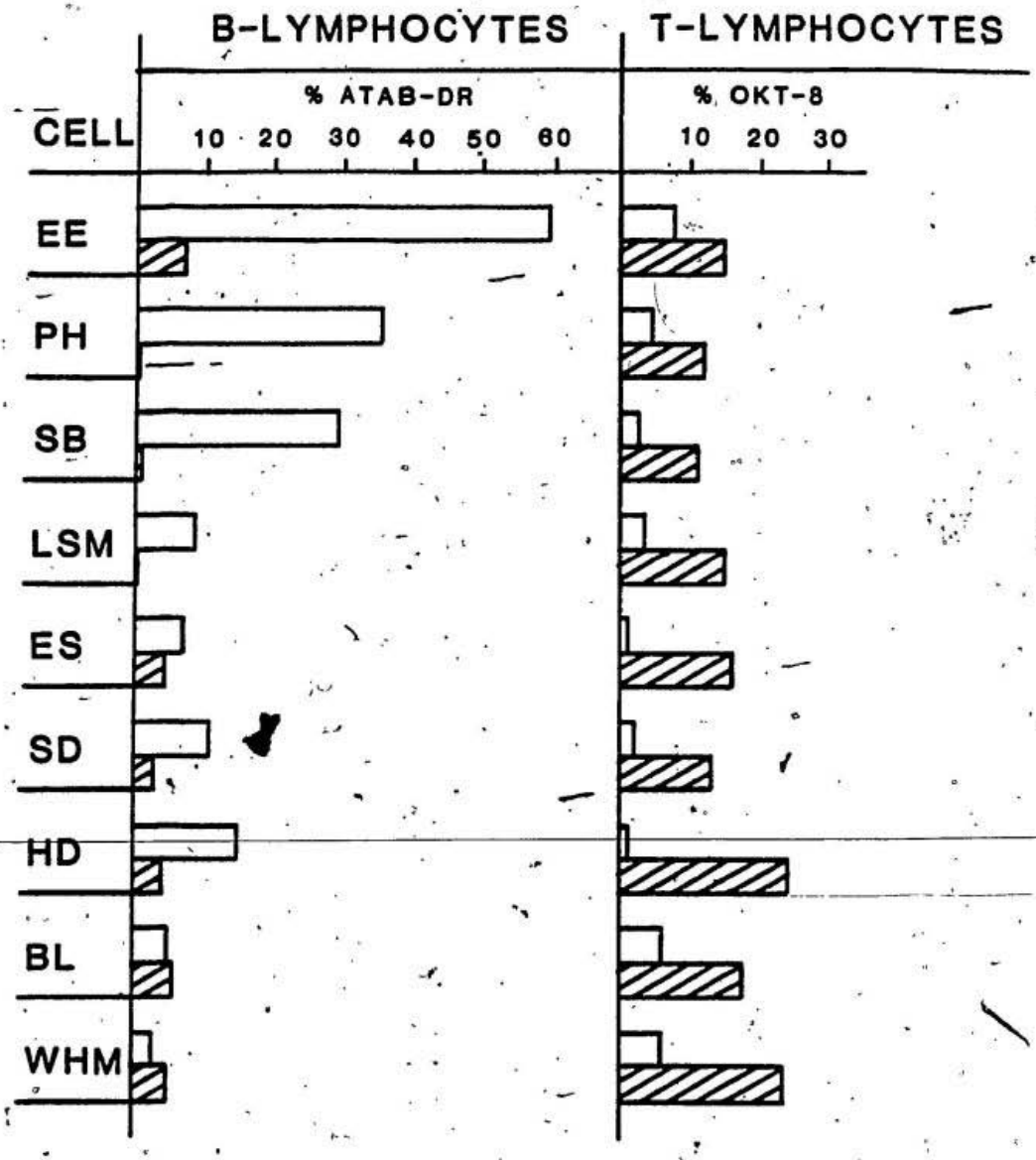

Figure 14. Efficiency of separating PBL into T- and B-lymphocytes as measured in CELISA and reaction of NFLD.M1 with the separated cells. The Adj. OD of each control MCAB + each cell population expressed as a percentage of the appropriate positive control (hatched bars). NFLD.M1 (open bars) reacted strongly with three B-cell preparations and none of the T-cell preparations.

expressed as a percent of the Adj. OD value for the ATAB-DR + B-lymphocyte combination from the same donor, the values (hatched bars) obtained for all nine sets were very low (range = 0 - 6.6%; X = 3.3; s = 2.2). This suggested minimal contamination since one would not expect OKT-8 to react with the B-lymphocyte population unless they were contaminated by T-lymphocytes.

The T-lymphocyte populations were analyzed similarly, using the Adj.OD value for ATAB-DR + T-lymphocytes expressed as a percent of the Adj. OD for OKT-8 + T-lymphocytes of the same donor. The values (hatched bars) were generally higher and more variable (range 11.1 - 24.1%; X = 16.4%; s = 4.7) than the B-lymphocyte results. This suggested that the T-lymphocytes were contaminated with DR-positive cells since ATAB-DR reacts with B-lymphocytes, monocytes and activated T-cells, all of which are DR-positive.

3.6.1B NFLD.M1 reactivity with T- and B-lymphocytes

Each of the Adj. OD values for NFLD.M1B + B-lymphocytes or T-lymphocytes was expressed as a percent of the Adj. OD value for the appropriate positive control, ATAB-DR + B cells and OKT-8 + T-cells (Figure 14, open bars). None of the T-cells reacted significantly with NFLD.M1B (range = 0.1-7.4% of OKT-8 control; X = 3.0% s = 2.1%). B-lymphocytes from EE, PH and SB reacted with NFLD.M1B, 29.1%, 36.2%, and 29.0% of the ATAB-DR control, respectively. B-lymphocytes from HD gave

an intermediate reaction (15.0%) and the remaining five were considered negative, ($\bar{X}$ = 6.6%, s = 2.8%). The mean and standard deviation for all 9 B-cell preparations against NFLD.M1B were 19.2% and 18.8% respectively.

3.6.1C NFLD.M1A titration on B-Lymphocytes:

The data from the titrations of NFLD.M1A and Freunds ascites against B-lymphocytes from the same donors (data not shown in Table 7B) confirmed that the only cells strongly reactive with NFLD.M1 were those derived from DR4 positive individuals (Table 7A and Figure 15). B-lymphocytes from HD (DRw6,w8), which had given an intermediate reaction with the supernatant (Figure 14), produced a similarly intermediate reaction with the NFLD.M1 ascites fluid and titered to 1/400. The DR4+ cells (EE, PH, and SB) were still reactive at 1/1600. This suggested that NFLD.M1 was reacting weakly with either DRw6 or DRw8 or both. None of the other B-cells reacted with NFLD.M1 to any greater degree than they did with the irrelevant ascites.

3.6.1D NFLD.M1 reactivity with PHA-blasts

Since DR molecules are expressed on the surface of activated T-lymphocytes but not resting T-cells (Brodsky and Radka 1985) one would expect NFLD.M1, if it recognizes a DR polymorphism, to react with activated T-cells. Therefore, NFLD.M1B (supernatant) was tested on PHA-blasts produced from

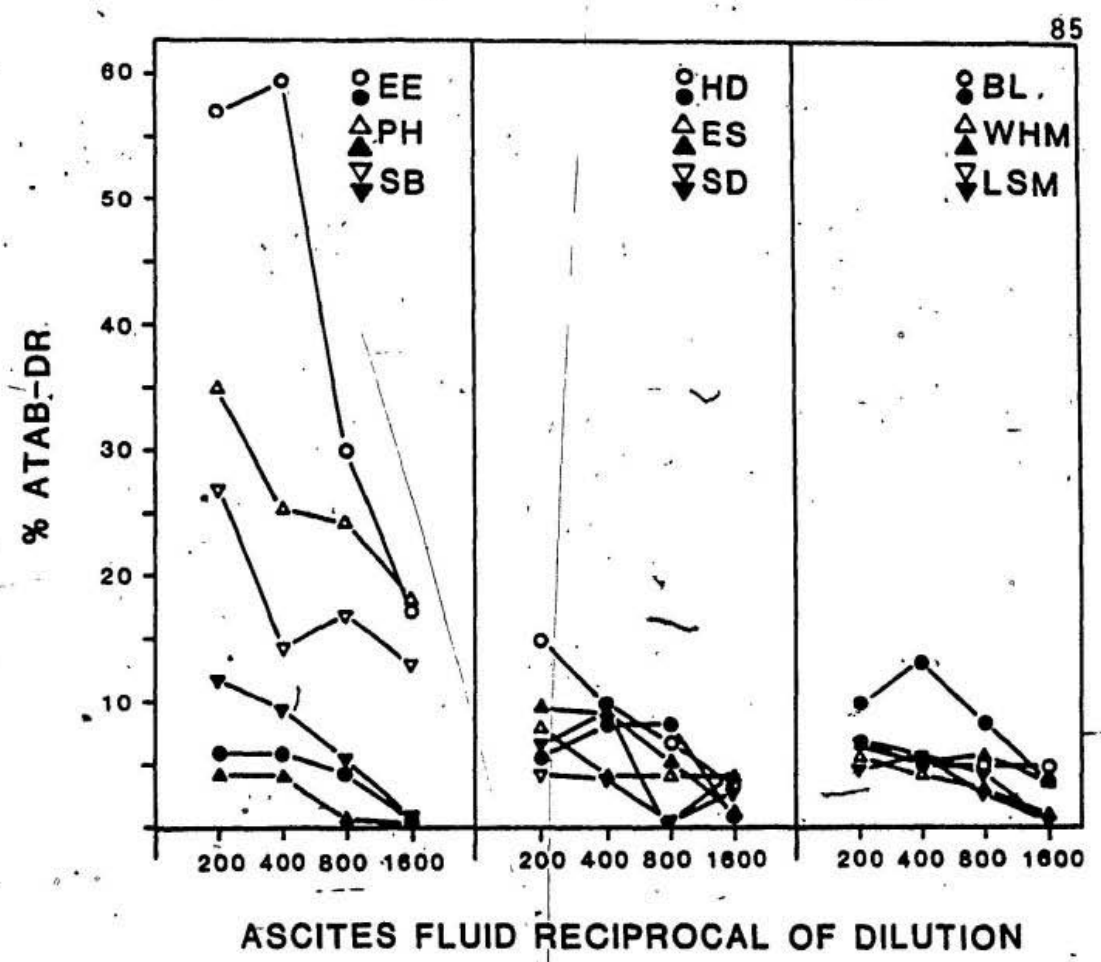

Figure 15. Reaction of specific and non-specific ascites fluid with B-lymphocytes from 9 unrelated individuals. NFLD.-M1B (open symbols) and Freunds ascites (filled symbols) were titrated against the cells in CELISA. The results are expressed as a percent of ATAB-DR, 1/800.

the same pool as the resting T-lymphocytes. The reactivity of ATAB-DR, OKT-8 and NFLD.M1 with PHA-blasts was assessed by comparison to the reactions obtained with resting T-cells (Figure 16).

The results supported the hypothesis that NFLD.M1 shows specificity for a DR determinant. Only those PHA-blasts obtained from the same three donors whose B-lymphocytes gave positive reactions with NFLD.M1, also showed strong reactions with NFLD.M1 ($\bar{X}$ = 554%; s = 516%). PHA-blasts from the remaining six gave a relatively small increase in NFLD.M1 reactivity ($\bar{X}$ = 61%; s = 58%). It is interesting that PHA-blasts from HD whose B-cells had given an intermediate reaction with NFLD.M1 were actually less reactive than were resting T- lymphocytes from the same donor. All PHA-blasts expressed substantially greater reactions with ATAB-DR ($\bar{X}$ = 404%; s = 156%) than with the resting T-cells, thus confirming that activated T-cells express DR and suggesting that the increased NFLD.M1 reactivity was related to DR expression. T8 expression was increased less dramatically ($\bar{X}$ = 181%, s = 36%) which is not surprising since it is also expressed on resting T-cells.

3.6.2 FAMILY DATA

To find whether the determinant recognized by NFLD.M1 was MHC-linked and inherited in a Mendelian fashion, T- and

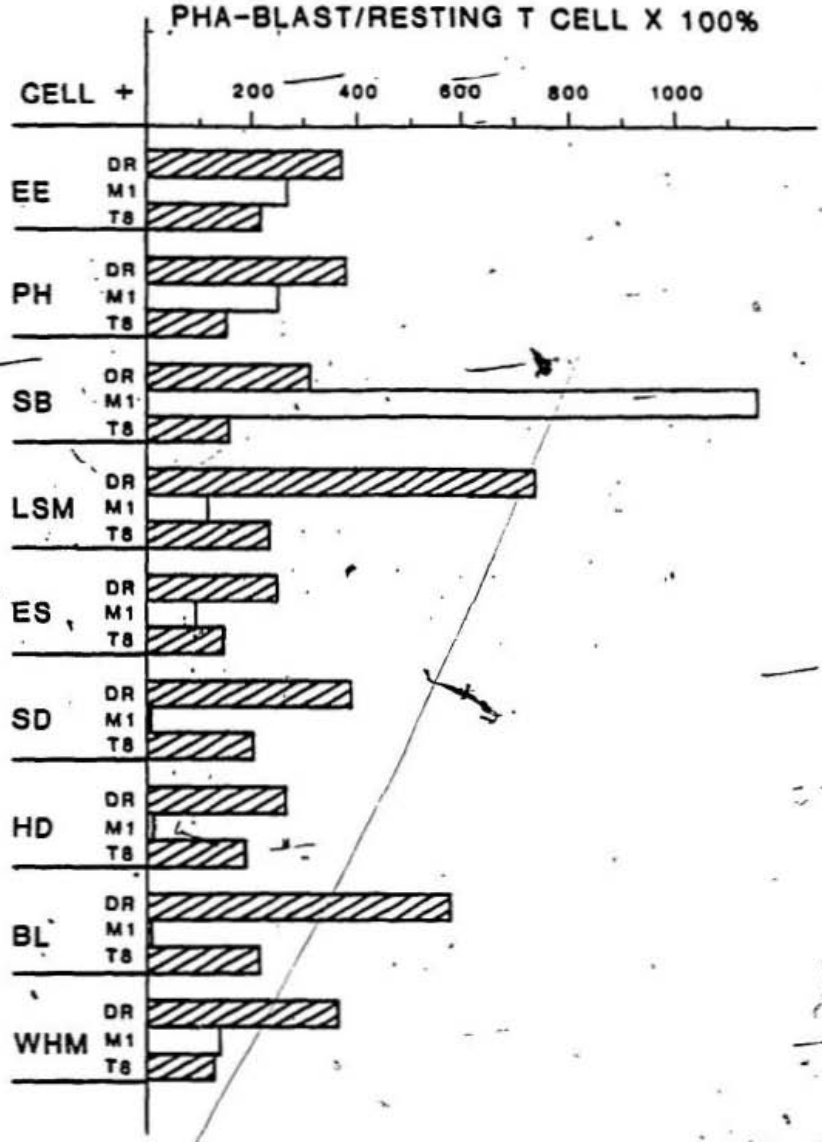

Figure 16. Effect of PHA-stimulation of T-cells on their reactions with MCAB. PHA-blasts derived from the same population as the resting T-cells were tested with each MCAB in CELISA. The Adj. OD value for each MCAB was expressed as a percent of the Adj. OD obtained for the same MCAB when equal numbers of resting T-cells were used.

+ DR = ATAB-DR (1/800); M1 = NFLD.M1 (SN); T8 = OKT-8 (1/100)

B-lymphocytes from 20 members of 4 families were reacted with NFLD.M1. ATAB-DR and OKT-3 MCAB were included as positive controls in order to evaluate the purity of each cell population.

3.6.2A Quality of the data.

The data for the B-lymphocyte preparations were generally better than those for the T-lymphocyte preparations. The OD values for ATAB-DR + B-lymphocytes were always greater than 3 times background for each cell ($\bar{X} = 5.0$, $s = 2.3$), whereas the OD values for OKT-3 + T-lymphocytes were sometimes less than 3 times background ($\bar{X} = 3.4$, $s = 1.4$). The purity of the B- and T-lymphocyte preparations was ascertained in the same way as described in section 3.6.1A. It is apparent from the data displayed in Figures 17A-17D that some T-lymphocyte preparations were either contaminated with B-lymphocytes or that the T-lymphocytes were activated since there was evidence of DR expression in some of these populations ($\bar{X} = 36.5\%$, $s = 18.2\%$). However, T-lymphocyte contamination of B-lymphocyte preparations was generally lower but more variable ($\bar{X} = 13.9\%$, $s = 18.2\%$).

3.6.2B Interpretation of Data

Families 251 and 502 were potentially informative for HLA-DR4 and NFLD.M1 (Figures 17A and 17B). All individuals of Family 504 had a DR4+ haplotype, and although not informative

Figure 17A-17D. Reactions of MCAB with cells from family members. B- and T-lymphocytes from HLA-haplotyped members of four families were tested with NFLD.M1 (SN), ATAB-DR (1/100) and OKT-3 (1/100) in CELISA. The Adj. OD value for each MCAB + each cell was expressed as a percent of the appropriate positive control.

B-lymphocytes:

□ = NFLD.M1/ATAB-DR

▨ = OKT-3/ATAB-DR

T-lymphocytes:

□ = NFLD.M1/ATAB-DR

▨ = ATAB-DR/OKT-3

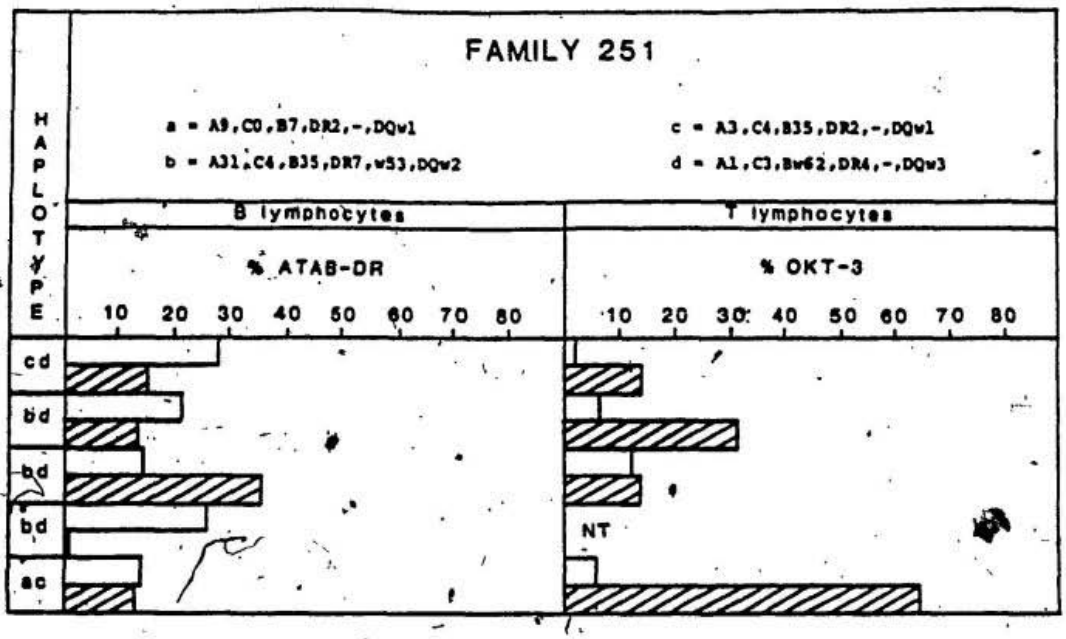

Figure 17A

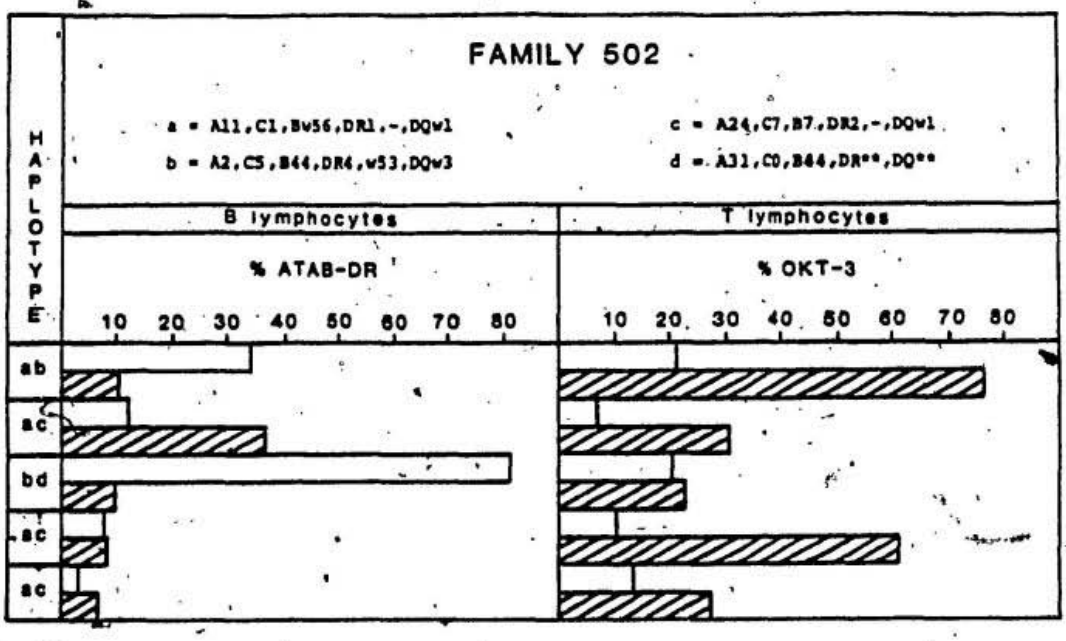

Figure 17B

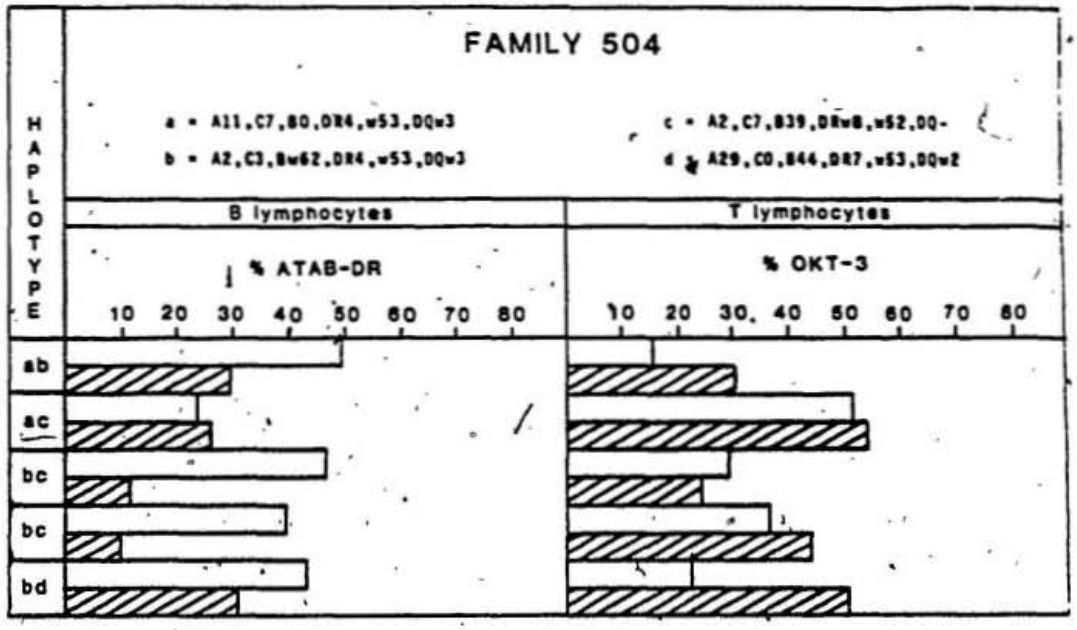

Figure 17C

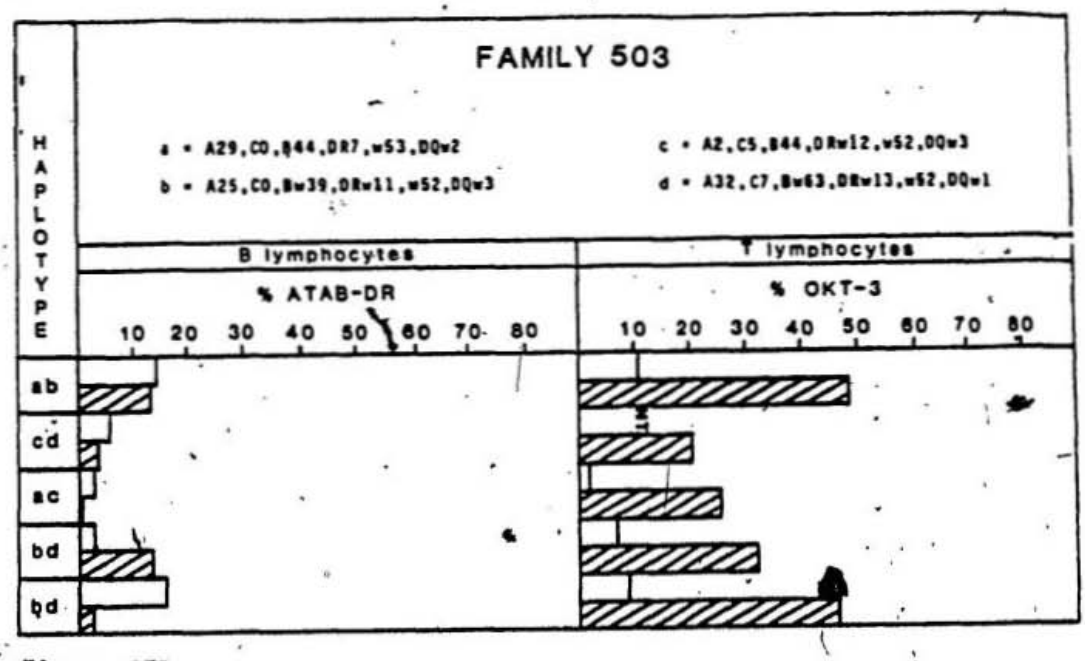

Figure 17D

for linkage, they were useful in confirming the DR4 and NFLD.M1 association (Figure 17C). Family 503 did not have a DR4+ haplotype but did have DRw53+DQw3+ cells and DRw53-DQw3+ cells (Figure 17D). Therefore, if NFLD.M1 were associated with one of these molecules then cells from this family would be very informative.

The reactivity of NFLD.M1 with the DR4 positive B-lymphocytes in Family 251 was unexpectedly low, and with the exception of the third individual (genotyped bd), this could not be explained by T-lymphocyte contamination. However, the d haplotype in this family is unusual because it lacks DRw53, which was found in association with 97.4% of DR4+ cells analyzed in the Ninth International Histocompatibility Workshop (LePage et al. 1984). There was no reaction of NFLD.M1 with the T-cell fractions.

Family 502 was the most informative but unfortunately, the maternal B-lymphocytes (genotyped cd) were not DR-typed, so it not known for certain if bd, homozygous at the HLA-ACB loci, is also homozygous for DR4,w53,DQw3. These cells gave the strongest reaction with NFLD.MI, 80% reactivity as compared to 35% reactivity with the paternal cells. T-lymphocytes with the same haplotypes gave an intermediate reaction with NFLD.M1. This may possibly be due to increased DR expression on the T-lymphocytes or B-lymphocyte contamination since ATAB-DR reacted significantly with these cells.

In Family 504 (Figure 17C) all cells expressed DR4,w53,

DQw3 and all reacted with NFLD.M1. However, some of these reactions were probably depressed due to impure preparations. The positive reactions of NFLD.M1 with the T-lymphocytes were most likely a reflection of increased expression of DR on these cells or impure preparations.

None of the haplotypes in Family 503 included DR4, but did include DRw53 and DQw3. The B-lymphocytes which appeared to have minimal T-cell contamination, were negative for NFLD.M1 and the T-lymphocyte suspensions, all of which reacted with ATAB-DR, were also negative for NFLD.M1.

The family data taken altogether were compatible with the idea that NFLD.M1 reacted with DR4+DRw53+DQw3+ cells, but not with DRw4-DR53+ cells, nor with DQw3+DR4- cells. In Family 502 the reactions of NFLD.M1 appeared to segregate with the DR4,w53,DQ3 haplotype. All HLA-ACB antigens known to be expressed on the immunizing cells were represented in this set of data. Since NFLD.M1 did not react satisfactorily with any of the T-lymphocytes, it is unlikely that a Class'1 epitope is being recognized by NFLD.M1. However, in view of the poor quality of the T-cell data this is stated with a certain degree of caution.

3.7 Serological Reactivity on B-Cell Lines

The serological pattern of NFLD.M1 on the peripheral

blood B-cells was consistent with that of an HLA-DR4 antibody with very little crossreactivity. This contrasted with the extra reactions seen with some B-LCL, particularly GM3190 (DRw6) and GM3160 (DRW12) during the screening and selection procedures (Section 3.3 this chapter).

3.7.1 Titrations

To confirm the specificity and analyze the extra reactions, NFLD.M1 (purified from ascites fluid), NEI-Ia and an irrelevant mouse IgG1 were titrated using doubling dilutions (5 µg/ml to 0.3125 µg/ml) against a panel of 19 LCL which included HLA-DR antigens 1-w8. Five µg/ml was chosen as the maximum concentration because that amount appeared to saturate the determinants on most cells (Figure 13). The results of the titrations (Table 8) clearly showed that an irrelevant mouse IgG1, even at 5 µg/ml, did not bind to the cells non-specifically. NEI-Ia reacted in a concentration-dependent manner with all cells. NFLD.M1, however produced highly variable results as can be seen from the standard deviations (Table 8) which is what one would expect from an antibody recognizing a polymorphism.

3.7.1A NFLD.M1 titrations

There was a distinct separation between clear positive

Table 8. Statistical Analyses of Titrations

	NEI-Ia		IgG1		NFLD.M1	
[Antibody]	X*	s	X	s	X	s
5.000 µg/ml	1476	276	138	24	428	389
2.500 µg/ml	1337	256	159	25	364	352
1.250 µg/ml	1157	214	172	29	310	280
0.625 µg/ml	1023	187	154	26	242	189
0.313 µg/ml	731	138	138	21	247	144

* = OD $x10^{-3}$

(DR4+ cells) and negative reactions (DR7+ cells) in the NFLD.M1 titrations (compare Figure 18A with 18F). When the results of the titrations for three DR4+ B-LCL were plotted (Figure 18A), differences in the strength of the reactions of NFLD.M1 with each cell, particularly between GM 1905 and LICR-LON-HYM2 (LICR-) were apparent. Although both cells were typed HLA-DR4,w6;w52,w53; DQw1,w3, they may still be HLA-D dissimilar since both DR4 and DRw6 are known to have several different subtypes.

The separation between positive and negative cells was not as clear for DR4-5+ and DR4-6+ cells. The titrations against DR4-5+ and DR4-6+ lines (Figures 18B-18C) were not as strong as those observed for DR4+ cells; nor were they clearly negative even at 0.3125 ug/ml. Reactions with the remaining cells were weak with some reacting at high concentrations, but titering to values similar to those obtained for IgG1, (Figure 18D), whereas others were considered negative (Figures 18E-18F). The lowest reactions were obtained for DR7+ cells (Figure 18F).

The titrations were divided into three groups on the basis of their reactions with NFLD.M1: positive (DR4+), intermediate (DR4-DR5+/DR6+), and negative (DR4-DR5-DR6-). When the mean and standard deviation for each group was calculated, variation within each group was apparent, particularly in the DR4+ group, (Table 9). This was mainly due to the high reactivity of NFLD.M1 with GM 1905 (DR4,w6)

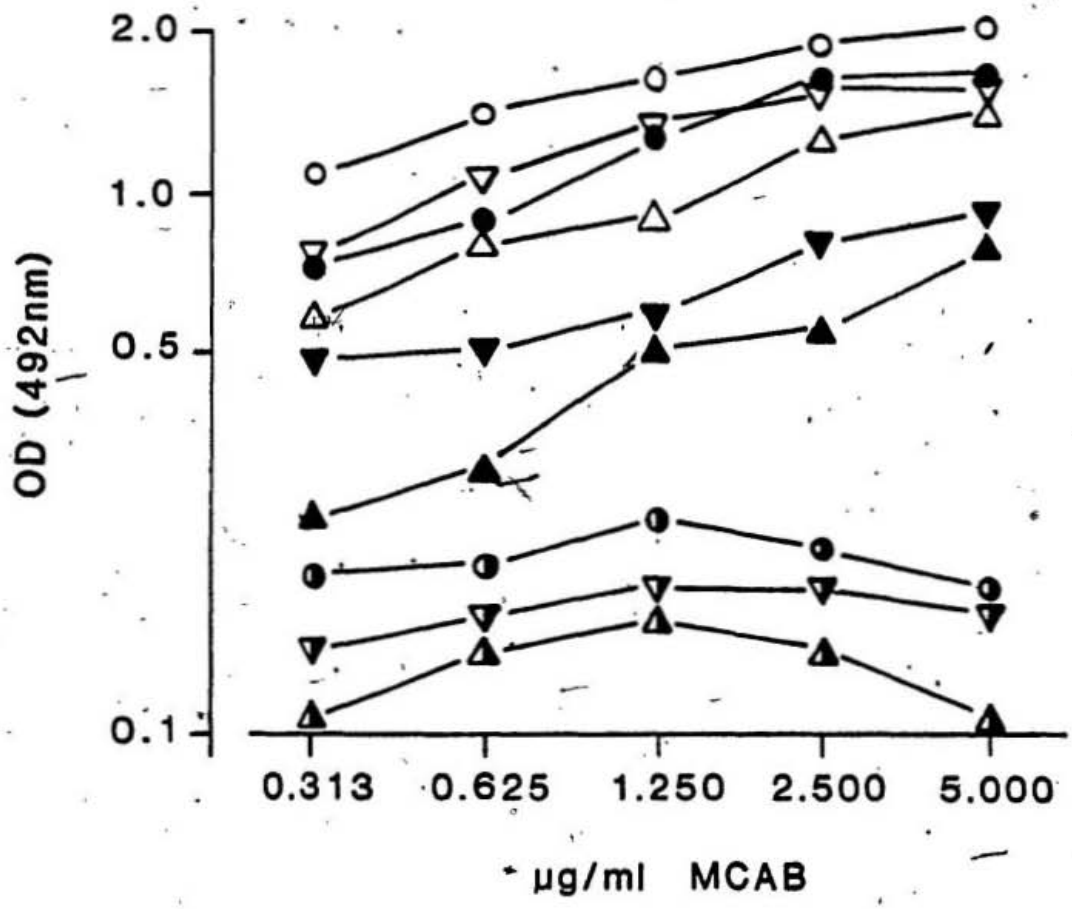

Figure 18A. Titrations of MCAB against DR4 positive B-LGL in CELISA:

	NFLD.M1	NEI-Ia	IgG1
GM1905 (DR4,w6)	●—●	○—○	●—●
GM1488 (DR4,5)	▼—▼	▽—▽	▼—▼
LICR- (DR4,w6)	▲—▲	△—△	▲—▲

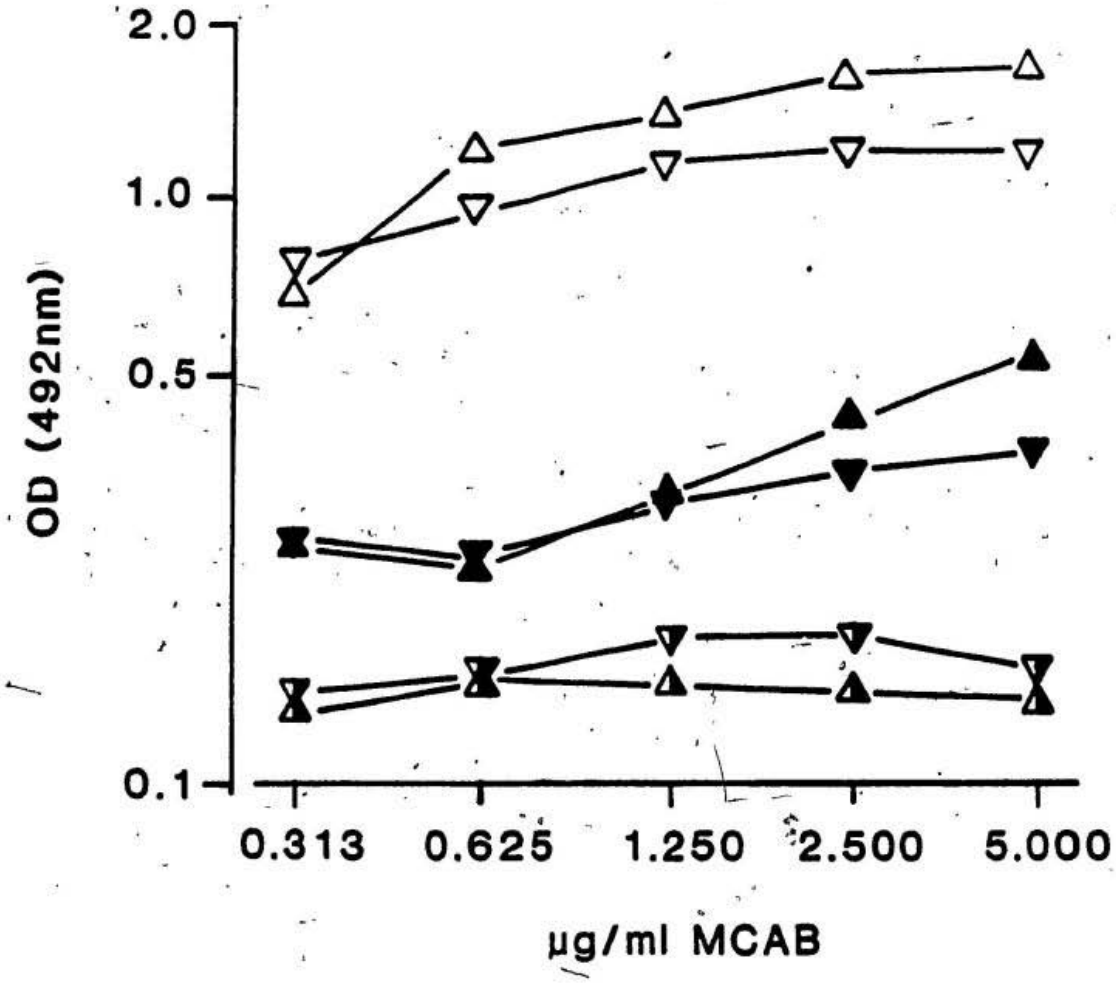

Figure 18B. Titrations of MCAB against DRw12 positive and DRw6 positive B-LCL in CELISA:

	NFLD.M1	NEI-Ia	IgG1
GM3160 (DRw12)	▲—▲	△—△	▲—▲
GM3190 (DRw6)	▼—▼	▽—▽	▼—▼

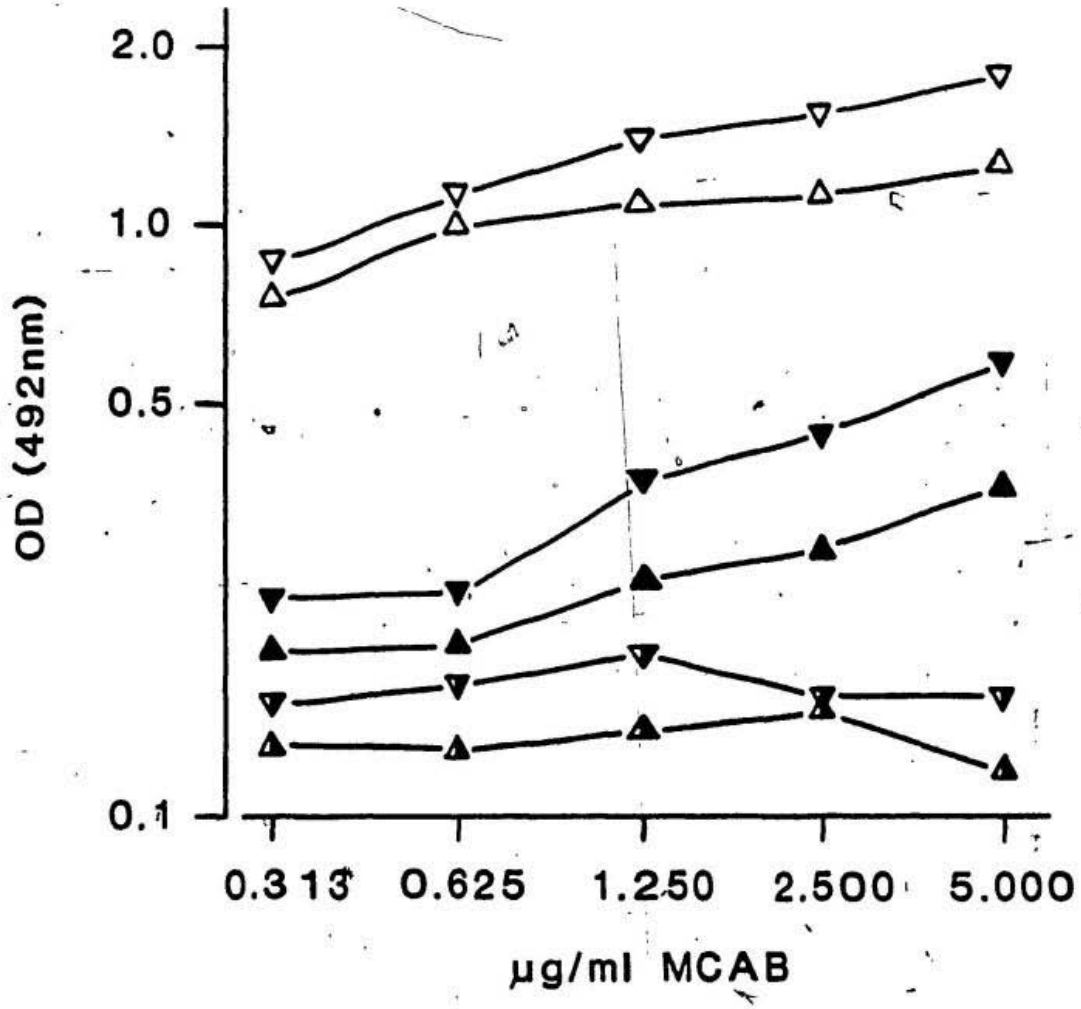

Figure 18C. Titrations of MCAB against DR5 positive B-LCL in CELISA:

	NFLD.M1	NEI-Ia	IgG1
GM3106 (DR5,5)	▼—▼	▽—▽	▼—▼
GM4672 (DR5)	▲—▲	△—△	▲—▲

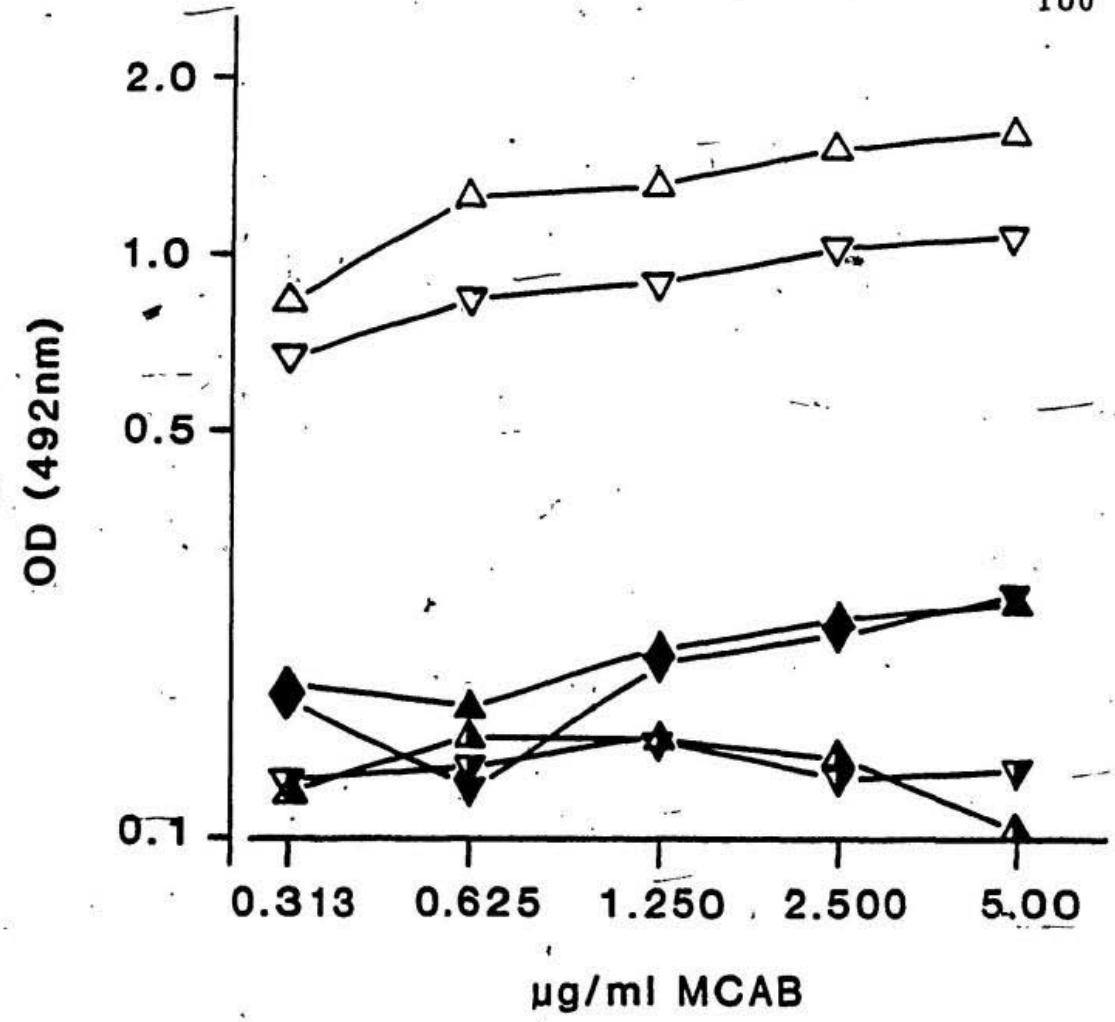

Figure 18D. Titrations of MCAB against DR2 positive B-LCL in CELISA:

	NFLD.M1	NEI-Ia	IGg1
GM1455 (DR2)	▼—▼	▽—▽	▼—▼
GM3161 (DR2,2)	▲—▲	△—△	▲—▲

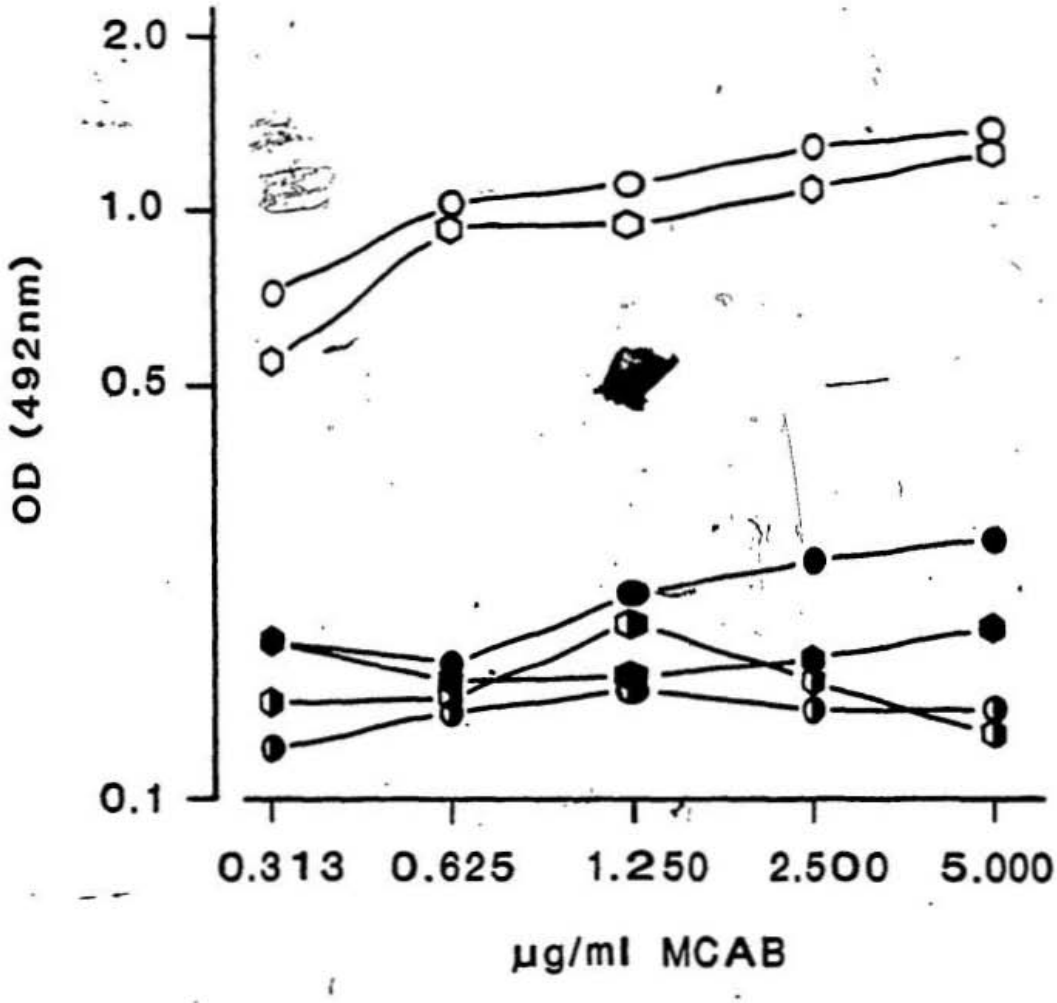

Figure 18E. Titrations of MCAB against DR3,7 positive and DR3,w8 positive B-LCL in CELISA:

	NFLD.M1	NEI-1a	IgG1
GM1032 (DR3,7)	●—●	○—○	⬢—⬢
GM1913 (DR3,w8)	●—●	○—○	●—●

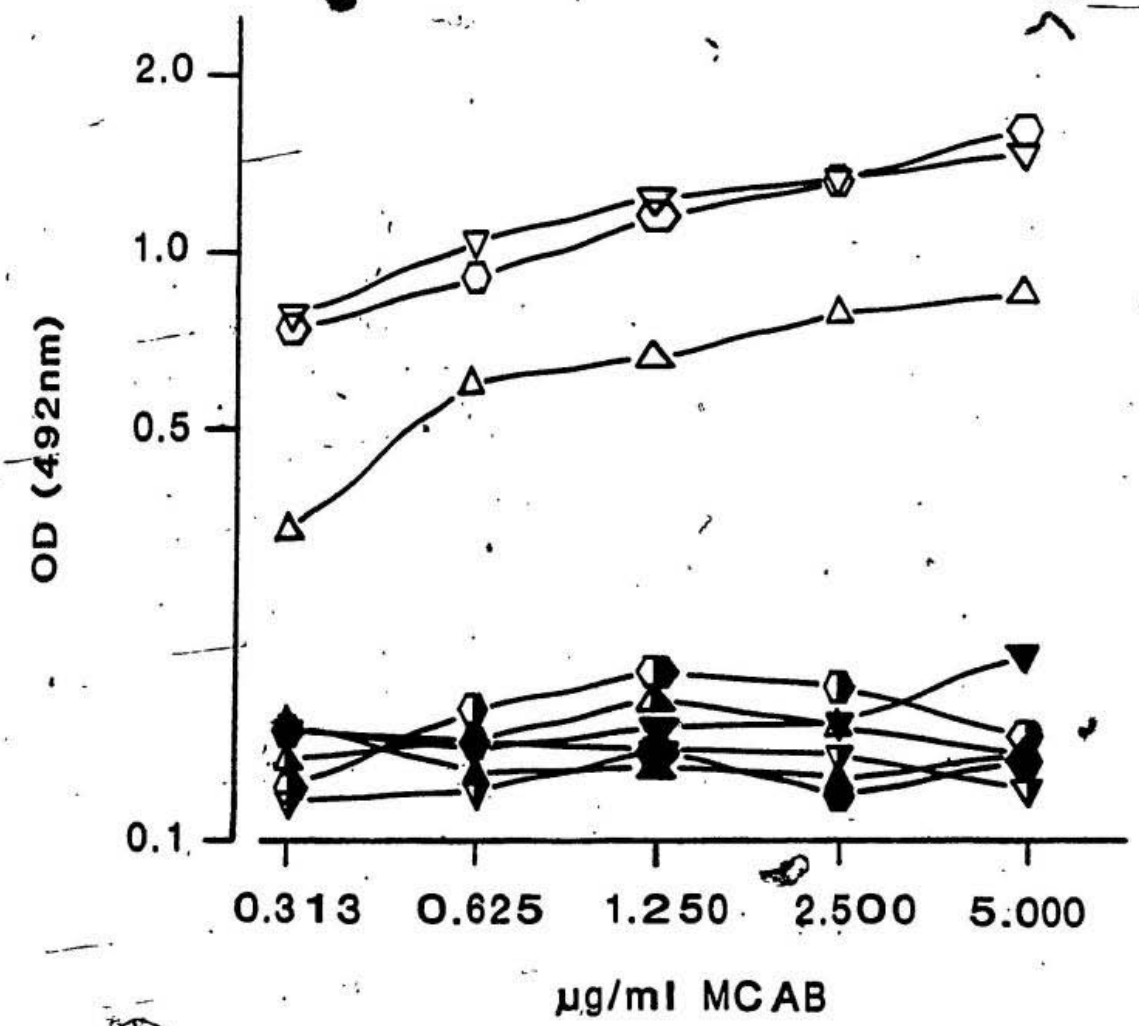

Figure 18F. Titrations of MCAB against DR1 positive and DR7 positive B-LCL in CELISA:

	NFLD.M1	NEI-Ia	IgG1
GM3104 (DR1,1)	▼—▼	▽—▽	▼—▼
GM3163 (DR7,7)	●—●	○—○	◐—◐
FS5059 (DR1,7)	▲—▲	△—△	◭—◭

Table 9. Statistical analyses of NFLD.MI titrations against three groups of cell lines in CELISA. The results are given as Adj.OD* values

	DR4+		DR5+/DR6+		OTHER	
[NFLD.MI] µg/ml	X	s	X	s	X	s
5.000	1172	405	453	112	217	64
2.500	1005	451	375	74	212	36
1.250	794	433	303	42	171	44
0.625	599	236	223	21	150	19
0.313	524	165	234	32	176	24

* OD $x10^3$

as discussed earlier in this section.

3.7.1B Relative reactivity of NFLD.M1 to NEI-Ia

The data for NFLD.M1 titrations were next analyzed relative to the NEI-Ia data for each of the three groups of cells. The relative reactivities for 14 titrations are given in Table 10A-C. The distinction between the DR4+DR5+/DR6+ cells and DR4-DR5+/DR6+ was more obvious then those displayed in Figures 18A-F.

3.7.2 Specificity testing on cell lines

Duplicate testing was performed on 42 B-LCL in CELISA with NFLD.M1B (purified from asoites fluid), and NEI--Ia using 50 ng MCAB and 5 x 10^4 cells/well. Culture medium was included as background control. The Adj. OD for each was calculated and if it varied from the mean by more than 15%, the test was rejected.

A Frequency Distribution of the Adj.OD values in Table 11 showed that the OD values for NEI-Ia reactions were normally distributed (Figure 19). The values ranged from 0.515 - 1.853 ($\bar{X}$ = 1.253; s = 0.307). Furthermore when the DR types are examined across this distribution, it is evident that there is no particular relationship to be seen between any DR type including DR4 and any part of the distribution of values. However, NFLD.M1 reactions were distributed bimodally

Table 10A. NFLD.M1 Reactivity with Group 1 Cells (DR4 positive)

	Cell lines				
HLA-	GM1905	LICR.	GM1488		
DR	4,6	4,6	4,5		
DR	52,53	52,53	52,53		
DQ	1,3	1,3	3		
[AB]					
ug/ml	% Reactivity*			X	s
5.000	84.9	56.3	60.0	66.1	13.8
2.500	80.4	44.5	54.6	59.8	18.5
1.250	75.4	55.1	39.9	56.9	17.8
0.625	69.6	35.6	37.8	47.7	19.2
0.313	59.6	44.1	51.4	51.7	7.8

* $\frac{\text{Adj. OD for NFLD.M1}}{\text{Adj. OD for NEI-Ia}} \times 100\%$

Table 10B. NFLD.M1 Reactivity with Group 2 cells (DR4 negative, DR5 or DRw6 positive)

	Cell lines					
HLA-	GM3190	GM3160	GM4672	GM3106		
DR	6,6	12,	5,	5,5		
DR	52	53	53	53		
DQ	1	3	3	1,3		
[AB] ug/ml	% REACTIVITY*				*X	2s
5.000	19.8	24.4	18.1	25.3	21.9	3.5
2.500	18.1	21.2	13.7	16.9	19.7	3.9
1.250	15.0	14.3	12.0	15.5	14.2	1.5
0.625	12.1	8.1	7.0	6.7	8.5	2.5
0.313	7.4	21.2	9.3	6.1	11.0	6.9

* $\frac{\text{Adj. OD for NFLD.M1}}{\text{Adj. OD for NEI-Ia}} \times 100\%$

Table 10C. NFLD.M1 reactivity with group 3 cells (DR4 negative, DR5 negative and DRw6 negative)

	Cell lines						
HLA-	GM3161	GM1455	GM1913	GM3104	GM3163	GM1032	FS5059
DR	2,2	2,8	3,8	1,1	7,7	3,7	1,7
DR	-	-	52,	-	53	52,53	53
DQ	1,1	1,	2,	1,1	2,2	2,2	1,2

Table 10C. continued

	Cell lines								
HLA-	GM3161	GM1455	GM1913	GM3104	GM3163	GM1032	FS5059		
[AB]									
ug/ml	% Reactivity*							X	s
5.000	11.6	13.1	11.3	7.1	0	3.5	0	6.7	5.6	2.500
7.5	10.0	10.9	2.9	0	1.1	0	4.6	4.7		
1.250	7.2	8.4	9.3	3.1	0	0.6	0	4.1	4.1	
0.625	3.2	1.3	3.8	1.7	0	0.5	0	1.5	1.7	0.313
7.4	7.3	8.3	4.9	0	6.7	3.6	5.5	2.9		

* $\frac{\text{Adj. OD for NFLD.M1}}{\text{Adj. OD for NEI-1a}} \times 100\%$

Table 11. CELISA results from testing 42 B-LCL against NFLD.M1 and NEI-Ia

Cell	HLA- DR	DR	DQ	Adj. OD NFLD.M1	NEI-Ia	%R*
GM3104	1,1		1,1	111	1428	7.8
GM1810	1,8	52	1,	127	1305	9.8
CN	1,3	N	1,2	7	597	13.2
CM	1,3	52	1,2	119	1017	11.7
FS5059	1,7	53	1,2	12	860	0
GM3161	2,2		1,1	186	1223	15.2
GM3107	2,2		1,1	306	1070	28.6
GM1861	2		1,3	244	1642	14.9
EC	2,		1	286	1467	19.5
EA	2,3	52	1,2	96	1027	9.3
MC	2,3	52	1,2	219	1526	14.4
FS3029	2,3	52	1,3	161	541	29.7
CAK	2	53	1,3	295	1010	29.2
SC	2	53	1,3	387	1466	26.4
GM1553	2,7	53	1,2	69	1101	6.3
DP	2,7	53	1,	154	1801	8.6
GM1455	2		1,3	143	827	17.3
LR	2,9	53	1,3	149	1853	8.0

Table 11. continued

Cell	DR	DR	DQ	NFLD.M1	NEI-Ia	%R
GM3098	3,3	52	2,2	242	914	26.4
MB	3,6	52	1,2	115	1469	7,8
GM1526	6,7	52,53	1,2	0	1387	0
GM1032	3,7	52,53	2,2	87	981	8.9
GM1913	3,8	52	2,3	206	1232	16.7
LLICRF	3,10	52	1,2	73	1841	4.0
ML	4,6	52,53	1,3	865	1109	78.0
TP	4,2	53	1,3	724	1171	61.8
AP	4,2	53	3,1	744	996	74.7
GM1559	4,2	53	3,1	802	1237	64.8
KH	4,3	53,52	3,2	795	1151	69.1
SB	4,3	53,52	3,2	632	1190	53.1
GM3356	4,5	53,52	3,3	781	913	85.6
GM1488	4,5	53,52	3,3	740	1330	55.6
BC	4,5	53,52	3,3	896	1588	56.4
GM1905	4,6	53,52	3,1	737	1331	55.4
L.LON	4,6	53,52	3,1	780	1363	57.2

Table 11. continued

Cell	DR	DR	DQ	NFLD.M1	NEI-Ia	%R
GM3105	5,5	52,52	3,3	159	1121	14.2
GM4672	5	52	3	229	1265	18.1
GM3160	12	52	3	434	1639	26.5
GM3190	6	52	1	342	1231	27.6
RB	6,7	52,53	1,2	92	1226	7.7
GM3163	7,7	53	2,2	-13	1600	0
KOZ	9,9	53	3,3	8	1587	5

* $\%R = \frac{\text{Adj. OD NFLD.M1}}{\text{Adj. OD NEI-Ia}} \times 100$

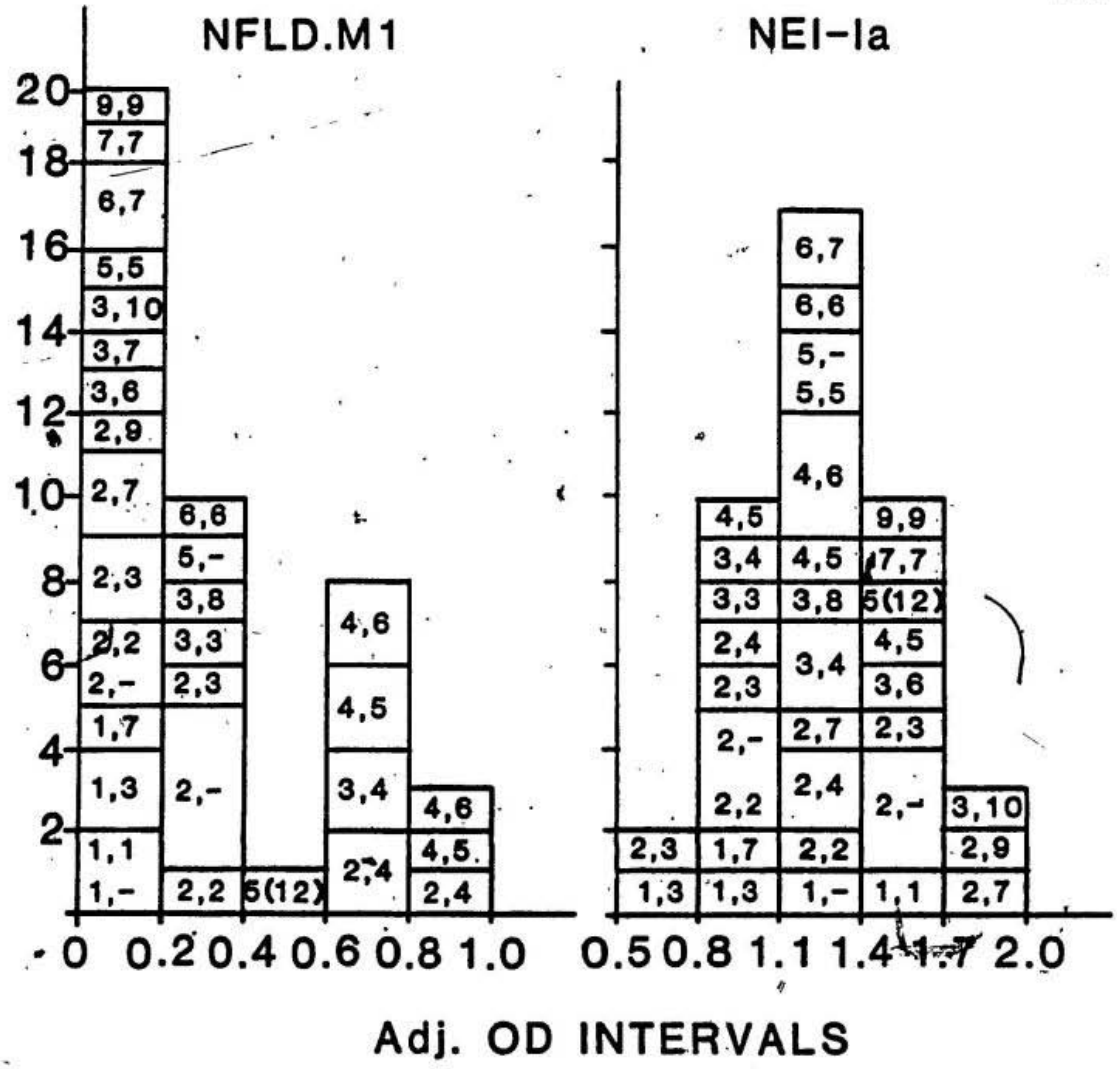

Figure 19. Frequency distribution plot of the Adj. OD values for 42 B-LCL tested against NEI-Ia and NFLD.M1. Each bar represents the number of cells in each interval and within each bar are the DR types of the cells.

with only one B-LCL producing a value in the overlapping interval. All cells in the high OD group were DR4 positive and all of the remaining cells were DR4 negative (Figure 19).

The bimodal distribution was even more sharply defined when the NFLD.M1 results expressed as a percentage of the NEI-Ia results with the same cells were plotted (Figure 20). Only cells expressing DR4 showed greater than 50% of NEI-Ia reactivity while DR4 negative cells showed less than 30% of NEI-Ia reactivity. Because no cell produced values in the intervals between 30% - 50%, 30% was chosen as a cutoff point for negative reactions. Heterogeneity was also apparent in the negative reactions and this will be discussed in Chapter IV.

3.8. Statistical Analysis of NFLD.M1

Using the 30% cutoff described above (Figure 20) and the data presented in Table 11 each of the 42 reactions was graded positive or negative. A correlation analysis between NFLD.M1 and each DR and DQ antigen was done as described in Section 2.6, Chapter II. The probability (p) was calculated using Fishers Exact test. NFLD.M1 was in complete concordance with DR4 ($P = 0.2 \times 10^{-9}$), (Table 12). In addition there was significant correlation with DRw53 ($r = 0.54$; $p = 0.3 \times 10^{-3}$) and DQw3 ($r = 0.57$; $p = 0.16 \times 10^{-3}$) but this was not

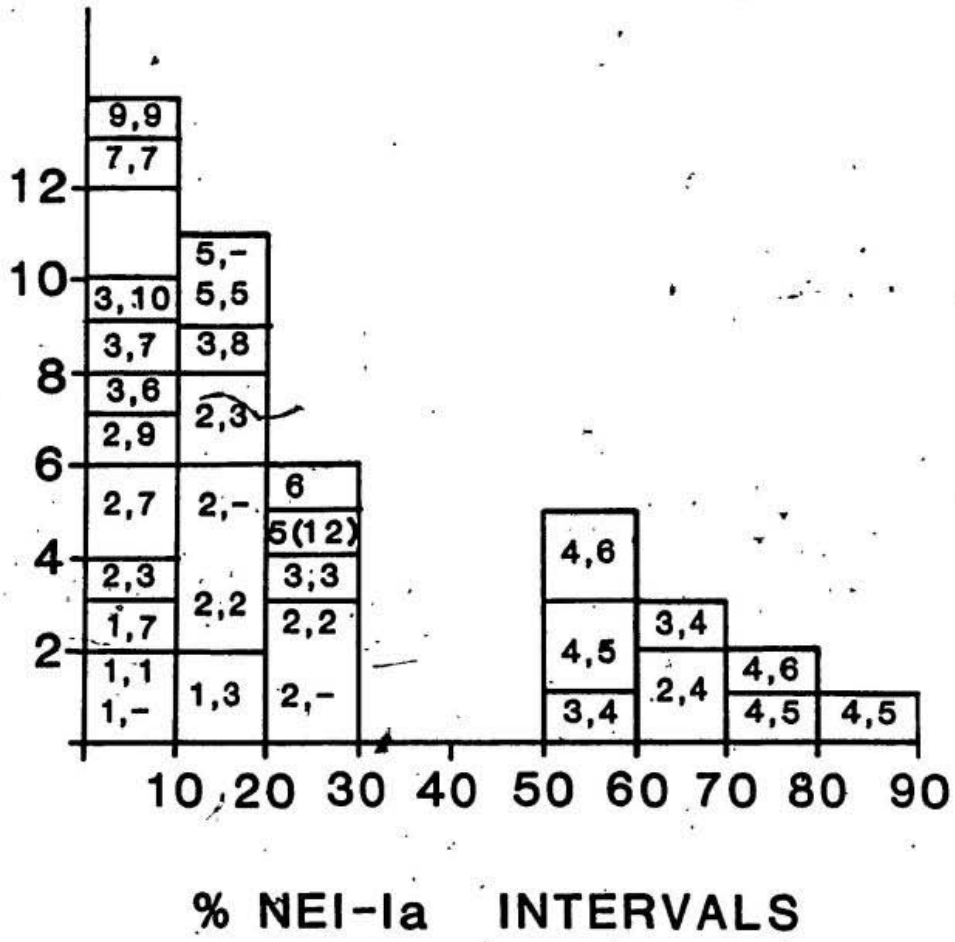

Figure 20. Frequency distribution plot of the % relative reactivity of NFLD.M1 tested against 42 B-LCL. The % relative reactivity (% NEI-Ia Intervals) is expressed as:

$$\frac{\text{Adj. OD NFLD.M1}}{\text{Adj. OD NEI-Ia}} \times 100$$

Each bar represents the number of cells in each interval and within each bar are the DR types of the cells. more clearly the bimodal distribution of NFLD.M1.

Table 12. Correlation of M1 with HLA-D Antigens

HLA-	++ a	+- b	-+ c	-- d	T	r	X^2	p
DR1	0	5	11	26	42	-0.22	0.77	1.2
DR2	3	13	8	18	42	-0.16	0.25	0.20
DR3	2	10	9	21	42	-0.42	0.25	0.22
DR4	11	0	0	31	42	+1.00	37.00	0.2×10^{-9}
DR5	3	3	8	28	42	+0.22	0.87	0.14
DR6	3	4	8	27	42	+0.17	0.39	0.19
DR7	0	7	11	24	42	-0.27	1.58	0.78
DR8	0	2	11	29	42	-0.13	0.002	1.62
DR9	0	2	11	29	42	-0.13	0.002	1.62
DR10	0	1	11	30	42	-0.09	0.003	1.48
DQ1	6	23	5	8	42	-0.19	0.69	0.14
DQw2	2	14	9	17	42	-0.24	1.49	0.088
DQw3	11	11	0	20	42	+0.57	11.10	0.2×10^{-3}
DRw52	8	16	3	15	42	+0.19	0.74	0.14
DRw53	11	12	0	19	42	+0.54	9.96	0.3×10^{-3}

unexpected since both these determinants are strongly associated with DR4.

3.9 Comparison of NFLD.M1A with NFLD.M1B

Testing of NFLD.M1 on peripheral blood B- and T-lymphocytes was done using supernatant obtained from the twice cloned culture (NFLD.M1B), whereas most of the testing on the LCL was done using 5 ug/ml purified antibody from an ascites induced with once cloned cells (NFLD.M1A). To test whether both were recognizing the same antigenic determinant, and also to see if the more concentrated M1B produced higher OD values both forms of antibody and NEI-Ia were tested in parallel on a panel of cell lines. The adjusted OD values of each NFLD.M1 test were expressed as a percentage of the adjusted NEI-Ia OD value (Table 13).

Because NFLD.M1 distribution was shown to be bimodal (Figures 19 and 20), the correlation coefficient (P) was calculated using Spearman's rank order method and the significance tested using the Student's t test as described in section 2.6, Chapter II. The results showed that overall there was real positive correlation between M1A and M1B in their serological reactivity on 23 LCL (significant t at 0.01, 2 tails, 21 degrees of freedom; = 2.831).

Table 13. Comparison of reactions of NFLD.M1A and NFLD.M1B with various cell lines in CELISA

Cell	DR	NEI-Ia OD[1]	M1A OD	M1A %R[2]	M1B OD	M1B %R
KOZ	9,9	1082	35	1.1	-7	0
GM1553	2,7	1093	69	6.3	31	2.8
LR	2,9	1724	149	8.6	149	8.6
DP	2,7	1801	154	8.6	79	4.4
CM	1,3	1017	119	11.7	70	6.9
CN	1,3	597	78	13.1	39	6.5
MC	2,3	1526	219	14.3	267	17.5
GM1032	2,3	678	132	15.4	47	5.5
EC	2,2	1487	282	19.0	277	18.6
GM3161	2,2	972	193	19.9	142	14.6
GM3160	5(12)	1177	239	20.3	293	24.9
GM1455	2,8	723	162	22.0	123	17.0
SC	2,5	1466	386	26.3	338	23.1
GM3098	3,3	914	250	27.4	176	19.3
FS3029	2,3	541	151	27.9	110	20.3
GM3107	2,2	1070	306	28.5	210	19.5
GM3190	6,6	1309	444	33.9	416	31.8

Table 13 continued

Cell	DR	NEI-Ia OD*	M1A OD	M1A %R	M1B OD	M1B %R
GM1905	4,6	1050	551	52,4	646	61.7
BC	4,5	1588	897	56.5	989	62.3
TP	2,4	1172	72.5	61.9	719	61.3
KH	3,4	1152	795	69.1	799	69.4
ML	4,-	1109	865	78.0	835	75.3
GM1559	2,4	1237	1102	89.0	813	65.6

1 Adj. OD x 10^3

2 %R = $\frac{\text{Adj. OD NFLD.M1-}}{\text{Adj. OD NEI-Ia}}$ x 100%

3.10 One Dimensional Gel Electrophoresis

NFLD.M1 and NEI-Ia were used to immunoprecipitate ^{125}I-labelled molecules from a cell lysate prepared from a DR4,6 cell line (GM 1905) followed by SDS-PAGE analysis and autoradiography as described in Chapter II. Each of the MCAB immunoprecipitated subunits that were in the molecular weight range of 27-34 Kd (Figure 21), which corresponds to the molecular weight of the beta and alpha subunits of the DR complex. A negative control monoclonal immunoglobulin (mouse IgG1) and an anti-human Ig antiserum did not precipitate similar complexes. The molecular weight markers showm in Figure 21 are BSA (67K), ovalbumin (43K), α chymotrypsin (26K) and β lactoglobulin (18.5K).

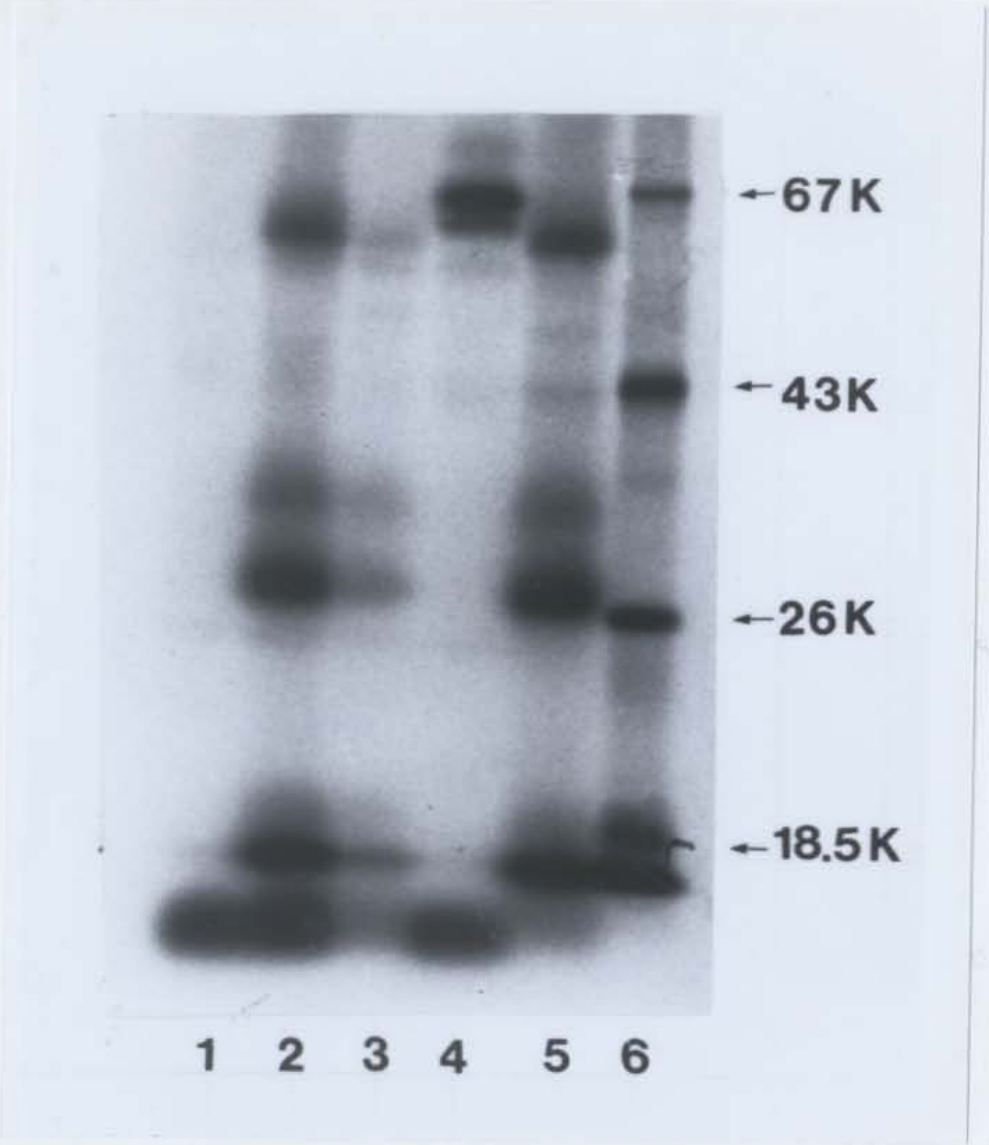

Figure 21. SDS-PAGE analysis of the immunoprecipitated molecules from ^{125}I labelled GM1905 cells. Lanes 2 and 5, NFLD.M1; lane 3, NEI anti-Ia; lanes 1 and 4, negative controls, murine IgG1 and anti-human IgG, respectively; lane 6, molecular weight markers.

CHAPTER IV DISCUSSION

4.1 Glutaraldehyde-fixation of Cells Induces Artifacts in ELISA for Testing Monoclonal Antibodies

The adaptation of an ELISA that used target cells stuck to plastic with poly-L-lysine and fixed with glutaraldehyde (Stocker and Heusser 1979; Suter et al. 1980; Cobbald and Waldmann 1981; Kennett 1981) at first seemed a good alternative to the radioimmune assay. However, it had to be abandoned in favor of a viable cell assay (CELISA) for two reasons: 1) the glutaraldehyde fixation caused irrelevant myeloma proteins to stick to the cells non-specifically, and 2) it modified or destroyed some HLA determinants.

Since non-specific binding was to the cells and not to the plastic, this suggests binding via Fc-receptors with perhaps some Fc-receptors being preferentially exposed by the glutaraldehyde treatment. Alternatively, it may have been caused by electrostatic binding. According to Emmrich (1985) the latter can be overcome by increasing the salt concentration in the wash buffers without losing sensitivity in the detection of low-affinity antibodies.

Assuming that there is a solution to non-specific binding, ELISA on GA-fixed cells would still be unacceptable

for testing hybridoma supernatants. The data presented in this thesis clearly shows that GA modifies and/or destroys some HLA-D region molecules. This effect of GA-fixation apparently results from modification of epsilon-amino, guanidine, secondary amino and hydroxyl groups of some amino acids with tyrosine, lysine and histidine being the most susceptible (Habeeb 1969; Hopgood 1972; Pancake and Nathenson 1973). As early as 1974 Gatti et al. reported that glutaraldehyde-fixation of murine cells results in selective impairment of H-2 antigens. This was confirmed by Bubbers and Henny (1975) who found that although GA-treated mouse cells still retain their antigenicity as measured by cytotoxicity and T-effector cells, they are unable to stimulate in MLC.

Several other reports deal with the effects of different concentrations and fixation times. For example, Baron et al. (1977) showed that there is a concentration-related destruction of HLA-D determinants with a 50% loss using 0.08% GA. They suggest using 0.24%, a concentration that was also used by Landsdorp et al. (1980). Other investigators have also urged caution with respect to the concentration and duration of glutaraldehyde-fixation (Van Ewijk et al. 1980; Liao et al. 1983; Beelan et al. 1984). A recent report by Walker et al. (1984) is especially relevant. Using monoclonal antibodies including NEI-Ia in several assays, they showed that irrespective of the species, tissues or cell types uses, fixation by formalin, glutaraldehyde, methanol, and ethanol

alters or destroys Class II antigens. The least damage is done when cell preparations are fixed quickly with cold acetone.

In contrast to these negative findings, there are several recent reports in which identification and characterization of HLA-D region monoclonal antibodies by ELISA on GA-fixed cells have been described. Holzmann and Johnson (1983) used an IgG3 monoclonal antibody to HLA-DR3 to analyze antigens on individual cells that had been fixed with glutaraldehyde. Shannon et al. (1984) using both RIA and ELISA on 0.25% GA-fixed cells identified and characterized an IgG1 monoclonal antibody to DQw3. In a slightly different system Bishari et al (1984) used alloantisera in an ELISA on GA-fixed cells, to distinguish between HLA class I homozygous and heterozygous cells.

It can be seen therefore, that there are numerous variables including the test systems, the source of antibody and antigen, the glutaraldehyde concentration and the duration of fixative. Glutaraldehyde does not appear to affect all antigenic determinants to the same extent; presumably this is related to the amino acid composition of the different determinants. Deleterious effects resulting from the fixation will be more obvious with monoclonal antibodies than with hyperimmune antisera because the mixture of polyclonal antibodies is less likely to reveal the obliteration of some epitopes on some molecules since some anti-

bodies may still bind to epitopes that have not been altered.

Finally, the possibility that unreacted aldehyde groups associated with the cell surface modifies the combining site of the antibody due to changes in the heavy chain should also be considered. It is particularly intriguing that most of the monoclonal antibodies described above that were successfully identified or characterized in ELISA using GA-fixed cells are all of the IgG1 or IgG3 subclass.

4.2 Assigning Specificity to NFLD.M1

The two sources of antibody, NFLD.M1A (purified from ascites fluid derived from the first clone, G3) and NFLD.M1B (overgrown supernatant from the subclone, H11), used for the specificity testing are most likely identical. Interpretation of the cloning results (Section 3.4, Chapter 3) using the Poisson distribution suggests that each was derived from a single cell. Additional confirmation of identity was obtained when both sources of antibody produced very similar curves when titrated against GM3190 cells (Figure 13). Parallel testing of both sources of antibody on several cell lines (Table 13) produced fairly conclusive evidence that both sources of antibody were reacting with the same epitope.

With the exception of a weak reaction with B cells from one individual (DRw6w8), neither source of NFLD.M1 reacted with any cells that were DR4 negative but each reacted with

all cells that were DR4 positive. The CELISA data on PHA-blasts derived from the same T-cells that were non-reactive with NFLD.M1, were compatible with the hypothesis that NFLD.M1 reacts with DR4 molecules. The only positive reactions were with PHA-blasts from the same individuals as were the three positive B-cells and all of these were DR4 positive. It is interesting that whereas B cells typed DRw6,w8; w52;DQw1 were moderately reactive with NFLD.M1, PHA-blasts from the same donor were clearly negative. This suggests that the B cell results may not have been due to reactions with DR molecules. It is possible, however that the reactions may have been due to DQ or DP molecules since both these molecules are only expressed on a subset of activated T-cells (Brodsky and Radka 1985).

The broad polymorphic pattern seen in the preliminary screening and selection procedures in which mainly B-LCL were used as targets (Figure 10) is in contrast to the DR4 association observed for NFLD.M1 tested with peripheral blood B cells (Figure 14). The CELISA results obtained when 42 B-LCL were tested with both NFLD.M1 and NEI-Ia (a monomorphic-DR) also confirm that there are reactions with some cells carrying antigens other than DR4 (Figures 18A-18F). Yet when reactions from all 42 cells were examined in a Frequency distribution plot, it is immediately obvious that NFLD.M1 reactions are clearly bimodal (Figures 19 and 20). Only DR4 positive cells are found in the high distribution (50-90%)

with not a single DR4 positive cell in the low (0-30%) distribution. For this reason 30% was used as a cutoff point to separate negative from positive reactions.

4.2.1 NFLD.M1 and DR4 specificity

There is complete concordance of NFLD.M1 with the DR4 positive cell lines tested. However, since the DR4 cell lines used to test NFLD.M1 were not MLC-typed, it cannot be concluded that NFLD.M1 recognizes a common epitope on all DR4 subtypes. HLA-DR4 comprises at least 5 different subtypes: Dw4, Dw10, Dw13 (DB3), Dw14 (LD40) and Dw15 (DYT) (Grosse-Wilde et al. 1984; Jaraquemada et al. 1984). The subtypes have been identified using MLC typing (Reinsmoen and Bach, 1982; Thompsen et al. 1983) and immunochemical analysis in which alloantibodies and/or monoclonal antibodies are used to immunoprecipitate D-region molecules followed by 2-D electrophoresis (Gronér et al. 1983; Nepom et al, 1983; Maeda et al. 1984). The polymorphic differences that were detected in MLC have been related to structural differences in the DR4 beta chain and to a lesser extent to structural differences in the DQw3 beta chain. Nepom et al. (1983) found five polypeptides that corresponded to DR beta chain variation and three polypeptides that corresponded to DQ variation. Al-

though Nepom et al. (1983), using neuraminidase-treated cells showed that Dw4 differed from Dw14 (LD40) only by the oligosaccharide differences, this could not be confirmed by Igarashi et al. (1984) who used tunicamycin to inhibit glycoslyation. Analysis of the amino-terminal part of the polypeptide from Dw4, Dw14 and Dw10 using high performance liquid chromatography suggests that the difference are due to the polypeptide structure and not oligosaccharide differences (Bach et al. 1984). Recently, Holbeck et al. (1985) using Southern blot analysis, have shown that DR4 is even more complex at the DNA level than at the protein level.

There are three serologically-defined subtypes, DR4.1, DR4.2 and DR4.3. This classification is based on serological patterns that emerged out of the Ninth International Workshop data (Williamson et al. 1984; Dawkins et al. 1984).
The DR4.1 subtype correlates with Dw13 and 40% of Dw4 subtypes, DR4.2 with Dw14 and 60% of Dw4 subtypes and DR4.3 with Dw15 subtypes (Noreen et al. 1985). Eventually, NFLD.M1 will be analyzed on homozygous DR4 lines that have been MLC-typed as well as typed for the serologically-defined subtypes.

4.2.2 Association of NFLD.M1 with DRw53

Since DRw53 is closely associated with DR4, the significant correlation between this specificity and NFLD.M1 is not

unexpected. The data obtained from the serological analysis in the 1984 Histocompatibility Workshop showed that DRw53 is present on approximately 98 percent of DR4+, 7+ and w9+ cells (LePage et al. 1984). Two dimensional gel analysis of molecules immunoprecipitated with alloantisera and monoclonal antibodies have shown that the DRw53 molecule is composed of the DR alpha chain and a beta chain that is distinct from the beta chains of DR4, DR7 and DRw9 molecules (Suzuki et al. 1984; Mukai et al. 1984; Maeda et al. 1986).

However, there is additional evidence that a DRw53 epitope on homozygous DR7 cell lines may reside also on the DR7 beta chain, or possibly on both beta chains (Toguchi et al. 1984). Knowles et al. (1984) and Horibe et al. (1984) using the DRw53-like monoclonal antibody (PL3) have performed 2-D gel analysis on immunoprecipitated molecules from a DR7 cell line have shown that the epitope recognized by this monoclonal antibody resides on the DR7 beta chain, whereas it resides on the second beta chain on the DR4 cells. It appears therefore that there are subtypes of DRw53.

Since neither of the two DR9+4- cell lines and none of the seven DR7+4- cell lines used in this study reacted with NFLD.M1, it is unlikely that NFLD.M1 reacts with the common serologically-defined DRw53 allodeterminant. However the possibility that NFLD.M1 recognizes a DRw53 epitope that is present on DR4 cells cannot be excluded. If this is the case, it may explain the low reactivity observed with the

53- B-cells of Family 251 (Figure 17A). The putative DRw53 epitope on the DR4 cells would therefore be structurally different from that present on the DRw9 and DR7 cell lines that were tested and which actually gave the lowest values of all 42 cell lines. This of course would have to be proven biochemically.

4.2.3 Association of NFLD.M1 with DQw3 cells

Like the association of NFLD.M1 with DRw53, the significant r and p values obtained for an association between NFLD.M1 and DQw3 are most likely related to the positive association between DQw3 and DR4 (Duquesnoy et al. 1984a). Of the six DQw3+DR4- cells that were negative for NFLD.M1, three were DR5+, one was DRw8+ and two were DRw9 positive. The results for the latter three lines were clearly negative, but the three DR5+ lines produced values at the upper end of the negative spectrum. The fact that NFLD.M1 did not react with PBL B-cells including cells from a family in which both DR5 subtypes (DRw11 and DRW12) and DQw3 were present suggests that NFLD.M1 does not react with DQw3.

It has recently been shown however, that DQw3 is a broad specificity that carries determinants on DR5+ cells that are different from those carried on DR4+ cells (Ishikawa et al. 1984; Tanigaki et al. 1984; Nunez et al. 1984; Giles and Capra 1985). Furthermore, there are monoclonal antibodies

which detect different DQw3 variants associated with DR4+ cells (Kasahara et al. 1983; Igarashi et al. 1984; Bontrop et al. 1986). Kim et al. (1985) using a monoclonal antibody to TA10 (DQw3.1) and restriction fragment length polymorphism (RFLP) typing have shown, that DR4+ cells with the same Dw specificity can have two different variants of DQ.

Therefore, if NFLD.M1 is associated with a DQw3 determinant that is only found on DR4 cells, it would be almost impossible to prove serologically. Immunochemical means would have to be employed.

4.2.4 Segregation of NFLD.M1 in families

By reacting NFLD.M1 with cells from members of four families, I had hoped to show that NFLD.M1 segregated in a Mendelian fashion with DR4 positive haplotypes. Although there were DR4 positive haplotypes in three families, the data was less informative than expected, mainly due to technical problems with some of the T-cell preparations.

One of the most intriguing findings was that the DR4 positive cells from the mother (haplotype d) in Family 251 were negative for DRw53 and produced reactions lower than expected with NFLD.M1 (Figure 17A). It is possible that the reactions between NFLD.M1 and DR4+ cells were low in this family due to the absence of DRw53 in this haplotype combination. Alternatively, NFLD.M1 may be recognizing a particular

DR4 subtype. Eventually, I hope to obtain more cells from this family, retest them in CELISA and analyze them immunochemically with NFLD.M1 and a DRw53 monoclonal made in this laboratory (Marshall and Drover, manuscript in preparation).

Although NFLD.M1 segregated with DR4 in Family 502 the evidence would have been more conclusive if the maternal B-cells had been DR-typed. Because all members of Family 504 carried a DR4 positive haplotype, it is impossible to say that NFLD.M1, which reacted with all of the cells, segregates with DR4. Members of Family 503, on the other hand were all negative for DR4 and NFLD.M1. Taken altogether, the family data confirm an association of NFLD.M1 with DR4 and reactivity with NFLD.M1 appears to segregate in a Mendelian fashion.

4.2.5 Reactions with DR4 negative cells

Although there is a clear delineation between the reactions of NFLD.M1 with DR4+ and DR4- cells, there are further reactions with certain LCL that are distributed in the first mode (<30%). For example, when the DR antigens of the cells that produced values of 15%-30% are considered, they are distributed as follows: 8/13 DR2, 3/10 DR3, 2/3 DR5, 1/4 DR6 and 1/2 DR8 cell lines. However, DR1 and DR7 as well as the two DR9 and the one DR10 LCL consistently produced

values less than 15%. This suggests that there is a determinant on the cell lines with 15%-30% reactivity that is reactive with NFLD.M1.

Although the nature of this reaction is unclear, the data obtained from the titrations of NFLD.M1 and IgG1 against cell lines (Figure 18A-18F) suggest that it is not via Fc receptors. However, Fc receptors cannot be eliminated unless testing is carried out using $F(ab)_2$ and Fc fragments prepared from NFLD.M1. Parham et al. (1984) found that Fab fragments, F(ab')2 fragments and /or the intact molecule can change the affinity of a monoclonal antibody for related epitopes.

Since most monoclonal antibodies are crossreactive (Colombani et al. 1984; Bodmer et al. 1984b), it would not be surprising if NFLD.M1 was also reacting weakly with a product encoded in a subregion other than DR. A monoclonal antibody may give extra reactions because it recognizes two similar but distinct epitopes on the same or different molecules or the same epitopes on different molecule (Parham 1984). Such an antibody can sometimes be made "operationally-specific" by diluting appropriately (Parham et. al. 1982; Sensi et al. 1985). However, when NFLD.M1 was titrated against several cell lines and compared to the NEI-Ia titrated against the same lines, the weak reactions observed with DR5 and DRw6 cells were not completely removed by dilution. This suggests that the extra reactions observed on these cells is not due to a crossreactive epitope. However, the extra reactions seen

with the other cells (DR2+, DR3+) decrease to background levels as the concentration of antibody decreases.

Since there were fewer extra reactions with the peripheral blood B cells than with the EBV-transformed cells, the possibility that NFLD.M1 crossreacts with an epitope unique to EBV-transformed B-cells should be considered. Cell lines are difficult to DR type accurately. This is usually explained by the presence of Class I antibodies in the antisera and the "enhanced sensitivity" of B-LCL to detect extra specificities due to an increased number of HLA molecules (Trucco et al. 1980). Recently, Stinchcombe et al. (1985) reported that 70% of their typings on B-LCL were discordant for HLA-DR and that one of their DR4 antisera reacted with a unique B-cell line antigen. An anti-DQw3 MCAB (2HB6) which was reported by Shannon et al (1984), also gave extra reactions with cell lines whereas it reacted primarily with DQw3+ cells when peripheral blood B-cells were tested. It is interesting to speculate that novel molecules, possibly related to class II molecules, may be expressed on EBV-transformed cells. If these putative molecules react with some DR/DQ antibodies, the results can be confusing.

4.3 Future plans for NFLD.M1

Although the data presented here shows that NFLD.M1

reacts with DR4 positive cell lines, whether it recognizes a broad or narrow DR4 specificity has not been shown. As stated earlier the DR4 specificity appears to be composed of several subtypes. Ideally NFLD.M1 should be tested with many homozygous DR4 cell lines with different Dw specificities as well as with many more peripheral blood B cells from unrelated individuals as well as families informative for DR4. If NFLD.M1 recognizes all DR4 molecules regardless of subtype, it would be similar to a DR4 'monospecific' alloantiserum and would be useful as a typing reagent particularly if an IgG2 switch variant were isolated (work in progress).

It would also be useful to do further serological analysis including affinity measurements (Mason et al. 1980 Parham 1984) using DR4+ cells as well as some of the cells that were weakly reactive. To find out whether the weak reactions are due to a classical antibody-antigen reaction or via Fc-receptors or some other mechanism, it would be necessary to prepare Fab fragments (Parham et al. 1982) or alternatively, remove the Fc receptors by protease digestion (Lobo et al. 1977). Inhibition studies, such as those described by Parham et al.1982) and Brodsky (1984), using 'monospecific' alloanti-DR4 serum and other available DR4 monoclonals would be helpful in sorting out the DR4 specificity and informative for epitope mapping.

Although 1-dimensional electrophoresis (Figure 21) is consistent with the immunoprecipitated molecules being DR

subunits, the evidence is very preliminary. Since the molecular weights of D-region molecules are similar, subtle differences would not be apparent. Two-dimensional gel electrophoresis of the immunoprecipitated molecules from homozygous DR4 cells and borderline positive B-LCL such as GM3190 and GM3160 would give further information about the antigenic structure of the NFLD.M1 determinant. I would especially like to EBV-transform B cells from informative members of Family 251 with the DR4+DRw53- haplotype and biochemically analyze the molecules immunoprecipitated with NFLD.M1 and other monoclonal antibodies.

Another very interesting possibility is to use this monoclonal to study disease associations. Disease associations with HLA-D region molecules have been extensively reviewed by Stastny et al. (1983). The association of HLA-DR4 with several autoimmune diseases including Type I diabetes and rheumatoid arthritis was confirmed during the Ninth International Workshop (Bertrams and Baur 1984; Christiansen et al. 1984). There is some evidence that different serological subtypes of DR4 are associated with different autoimmune diseases. The most common type, DR4.1 is found more frequently increased in rheumatoid arthritis whereas DR4.3 is significantly increased in Type I diabetes (Tait et al. 1984).

A recently defined HLA-D associated antigen, MC1 which shows strong linkage disequilibrium with DR4 and DR1 is also

associated with rheumatoid arthritis (Duquesnoy et al. 1984b and c). This is particularly interesting because DR4-negative rheumatoid arthritis may also be associated with DR1 (Christiansen et al. 1984). Another interesting finding is an RFLP pattern obtained with a DQ beta gene probe in the DNA from Type I diabetes cells, but not from that of rheumatoid arthritis. The pattern is concordant with the serologically defined DQw3.2 subtype (Kim et al. 1985).

In view of these recent findings I would like to purify DR4 molecules from several different Dw subtypes and raise monoclonal antibodies to different determinants in order to obtain monoclonals recognizing DR4 splits. These would undoubtedly prove useful in identifying DR4-specific epitopes, if indeed they exist for diseases such as Type I diabetes and rheumatoid arthritis. Such antibodies would also be useful for isolating DR4 molecules from cells of patients with Type I diabetes, rheumatoid arthritis or other diseases in order to biochemically analyze structural differences of disease-related epitopes.

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APPENDIX A

HLA Phenotypes of Cell Panel

CELL	A	C	B	DR	DR	DQ
AP[a]	2,3	N	18,54	2,4	53	1,3
BC	1,2	3,6	5,17	4,5	52,53	3
CAK	1,24	2,6	7,44	2	53	1?3
CM	1,2	3	8,40	1,3	52	1,2
CN	2,11	N	17,35	1,3	?	1,2
DP	2,30	3	7,60	2,7	53	1
EA	1,2	N	8,14	2,3	52	1,2
EC	1,3	6	7,57	2	N	1
KH	1,2	1	8,54	3,4	52,53	2,3
LR	3,24	1	7,15	2,9	53	1,3
MB	3,31	7	8,18	3,6	52	1,2
MC	1,24	4	7,18	2,3	52	1,2
ML	2,3	N	7,51	4,6	52,53	1,3
NB*	2,11	3,4	15,35	4	NT	NT
RB	3,30	6	13,18	6,7	52,53	1,2
SB	24,31	5,7	8,44	3,4	52,53	2,3
SC	3,25	N	7,44	2	53	1,3
TP	2,28	N	18,54	2,4	53	1,3
FS3029[ab]	1,2	N	8,14	2,3	52	1,3
FS3425[b]	2,25	N	7,18	3	NT	NT

APPENDIX A

HLA Phenotypes of Cell Panel

CELL	A	C	B	DR	DRw	DQ
FS4115[b]	2,28	3,5	4,40	4	NT	NT
FS5059[ab]	3	4,6	7,35	1,7	53	1,2
GM1032[c]	9,10	N	13	3/5,7	52,53	2
GM1455	11	N	22	2	NT	1,3
GM1488	26	N	16	4,5	52,53	3
GM1526	9,10	N	17,13	6,7	52,53	1,2
GM1553	28,3	N	22,13	2,7	53	1,2
GM1559	3,10	2	15,27	2,4	53	1,3
GM1810	1,10	N	7,13	1,8	52	1
GM1861	3,2	N	27,22	2	NT	1,3
GM1905	11	N	35	4,6	52,53	1,3
GM1913	2,31	N	18,13	3,8	52	2,3
GM2219	1,10	N	?	N	N	N
GM2131	2	N	7	4,7	NT	NT
GM3098	2,	N	17	3	52	2
GM3099	2	4	35	1	NT	NT
GM3103	9	N	14	4	53	3
GM3104	3	4	35	1	N	1
GM3105	26,28	N	38,18	5	52	1
GM3106	1	4	35	5	52	1
GM3107	3	N	7	2	N	1

APPENDIX A

HLA Phenotypes of Cell Panel

CELL	A	C	B	DR	DR	DQ
GM3160	2,	N	35,12	12	53	3
GM3161	3,	N	7	2	N	1
GM3162	2,	N	8,35	3	NT	NT
GM3163	30	6	13	7	53	2
GM3190	N	N	N	6	52	1
GM3356	2,10	N	16	4,5	52,53	3
GM4672	2,24	N	14,18	5	52	3
LLICRF[d]	1,2/28	7	8,35	3,10	52	1,2
KOZ[d]	24,26	1	40,54	9	53	3
KR4[e]	2	N	14,18	3,10	52	1,2
LICR-LON-HYM2[f]	2,?	4	15,35	4,6	52,53	1,3

* Immunizing cells

a = EBV-transformed cells prepared in the Immunology Laboratory

b = Non-transformed CLL cells

ab= Both EBV-transformed and non-transformed CLL cells used.

c = Cells with a GM prefix were obtained from the Human Genetic Mutant Cell Repository.

d = These cells were obtained from Dr. J. Bodmer, London, UK.

e = This cell is a fusion partner obtained from Dr. J.C. Roder, Kingston, Ontario.

f = This cell was obtained from Dr. M.J O'Hare, Ludwig Institute for Cancer Research, Surrey, UK.