THE EFFECT OF PRE-EXISTING ANTIBODY ON THE RESULT OF IMMUNIZATION OF MICE WITH HUMAN B CELL LINES AND TRANSFECTED MOUSE CELL LINES

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The Effect of Pre-Existing Antibody On the Result of Immunization of Mice With Human B Cell Lines And Transfected Mouse Cell Lines

by

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ABSTRACT

Henry and Jerne (1968) described an interesting phenomenon, when SRBC's were used as antigen, the presence of IgG antibody prior to injection of antigen led to a suppressed response. In contrast, pre-existing IgM resulted in an increase of the primary immune response. The aims of this project were twofold, to determine if the same phenomenon is observed when using either human B cells or mouse transfected fibroblasts, as antigens; to study the feasibility of using IgM enhancement to improve the yield of antigen-specific hybridomas from mice against HLA-DP and DR.

Studies were conducted using mice immunised either with a mouse transfectant cell line expressing HLA-DP (L25.4) or a human B cell line (RAJI). Preliminary experiments uncovered an unexpected reverse dose response when L25.4 cells were used as immunogen. Pre-treatment of mice with antibodies to monomorphic HLA-DP determinants increased and suppressed the responses as predicted; were statistically significant with both Raji and L25.4. In a second experiment, doubling of the IgM dose led to an unexpected diminution of the primary response against L25.4 and revealed that the half life of the administered IgM antibody appeared to be lengthened.

Experiments were then conducted to determine the value of IgM pretreatment in the creation of hybridomas specific for HLA-DP and HLA-DR epitopes. Mice were

pretreated with varying amounts of IgM antibody and then immunized. Three days later their spleens were removed, cells counted and fusions performed. Spleen size was found to be increased in a stepwise fashion with increasing doses of IgM past a previously described antigen induced plateau. The low dose of IgM produced spleens predominately composed of B cells, increasing the IgM dose decreased the B cells but increased the T cells. The IgM pretreated animals gave a 22.8% yield of specific hybridomas whereas the control group produced an 8%yield of specific hybridomas. Within the IgM pretreatment group, 96%of the selected hybrids originated from the low dosage group, indicating that there may exist an optimum pretreatment dose for IgM. These preliminary experiments support the potential of specific antibody pretreatment in the modulation of the primary response and the development of antigen-specific hybridomas.

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A	Adenine
BSA	Bovine serum albumin
CDC	Complement dependent cytotoxicity
CELISA	Cellular enzyme linked immunosorbent assay
D	Aspartic acid
DMEM	Dulbecco's modified eagle medium
E	Glutamic acid
EIA Microplates	Enzyme - linked immunoassay absorbent microplates
ELISA	Enzyme-linked immunosorbent assay
F	Phenylalanine
FACS	Flourescence activated cell sorter
FBS	Fetal bovine serum
GAM	Goat anti-mouse
HAT	Hypoxanthine-aminopterin-thymidine
HT	Hypoxanthine-thymidine
HLA	Human leucocyte antigen
HLA - DP	One of the six loci comprising the HLA "system"
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I/V	Intravenous
K	Lysine
L	Leucine
N.M.S	Normal mouse sera
O.D	Optical density
OPD	Orthophenylenediamine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
P/S	1% Penicillin and 1% Streptomycin
PLL	Poly - l - lysine
PVC Microplates	Polyvinyl chloride microplates
R	Arginine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRBC	Sheep red blood cell
TEMED	N, N, N' - tetramethylethylenediamine
TNB	Terra Nova Biotechnology
V	Valine
Y	Tyrosine

CHAPTER 1

INTRODUCTION

1.1 Human Leukocyte Antigen - DP Project

One of the requirements for successful transplantation is to ensure proper matching of the recipient and donor at the Major Histocompatibility Complex (MHC) level. The human MHC contains a group of highly polymorphic genes, products of which are called human leukocyte antigens (HLA) molecules and which are expressed on a variety of cell surfaces. There are two classes of MHC molecules, Class I and II. The Class II genes consist of DR, DQ and DP loci. DP is a fully functioning MHC molecule, presenting to T cells and showing MHC restriction. Until now the emphasis for tissue matching has been on the DR and DQ loci.

In a collaborative project that is presently underway, the aim has been to produce monoclonal antibodies that react with polymorphic determinants on HLA-DP molecules for use in tissue matching. The production of antibodies to the differing epitopes has proven to be a difficult undertaking, and it was suggested that it might be possible to make use of the manipulation of preexisting antibody levels to improve the immunisation schedules and consequently to increase the yield of hybridomas specific for epitopes of the DP molecules.

1.2 Basic Phenomenon Shown with Red Cells as Antigen

An interesting occurrence has been documented in the literature : when sheep red blood cells (SRBC) are used as an antigen, the presence of specific IgG antibody in the blood at the time of injection of antigen can cause the animal's response to be suppressed (Heyman,1990; Heyman,1988; Heyman *et al.* 1988; Quintana *et al.* 1987). For example one study reported more than 99% of the immune response was suppressed in such a manner (Sinclair,1978). On the other hand, pre-existing specific IgM (macroglobulin) at the time of antigen injection results in an increase in the primary immune response (Coulie and Snick,1985; Dennert,1971; Henry and Jerne,1968; Heyman *et al.* 1982; Heyman and Wigzell,1985; Heyman *et al.* 1988; Powell *et al.* 1982; Reiter *et al.* 1986). A practical example of the regulating capacity of pre-existing antibodies exists in humans as shown by the injection of IgG anti-rhesus D antibodies into Rh negative mothers at the time of delivery of their Rh^+ babies. This treatment has significantly reduced the frequency of iso-immunisation against the Rhesus D antigen and hence has decreased haemolytic disease of the newborn (Clarke *et al.* 1963). It was later shown by Pollack that antibody-mediated immune suppression mechanisms were responsible for this effect as opposed to simple clearance of the antigen antibody (Pollack *et al.* 1971).

1.3 Review of Literature Relating to Primary Immune Response Modulation

1.3.1 Early History

To understand how this line of investigation first started one can go back to the early 1900's. In 1909, Theobald Smith demonstrated that injection of partially neutralized diphtheria toxin-antitoxin mixtures induced antitoxin formation in experimental animals but that immunity did not result from mixtures containing a sufficient excess of antitoxin (Smith,1909). The results of these experiments were extended to the study of additional antigens such as tetanus toxoid (TT), sheep red blood cells and bacteriophages (Uhr and Baumann,1960). One of the many questions later raised from Smith's experiment was : Do pre-existing antibody levels have any effect on the result of immunisation?

It was not until the 1960's that significant work and results started to appear in the literature concerning this question. Investigators began to theorize and prove that pre-existing antibodies could regulate the primary immune response in both enhancing and suppressive ways.

1.3.2 Landmark Study by Claudia Henry & Niels Jerne

Claudia Henry and Niels Jerne, in 1968, investigated the idea of passively administered antibody having an effect on the primary immune response. The study used

in excess of 1000 animals, purified antibodies from a variety of methods, a variety of antigen and antibody doses and a variety of immunization time schedules. The focus of the experiments was to determine the effect of pre-existing 7S and 19S antibodies on the result of immunization of mice with SRBC on the primary immune response as measured by plaque assays. The results clearly showed that all 7S preparations suppressed the response while all 19S preparations enhanced the response. The IgM mediated increase was a fifteen fold enhancement in the number of plaque forming cells (Henry and Jerne, 1968). However, in earlier studies (Moller and Wigzell, 1965; Pearlman,1967), it was shown that IgM given at high doses (comparable to the high doses of the Henry and Jerne experiment) could be suppressive. The Henry and Jerne experiment (Figure 1) included a broad spectrum of doses of IgM antibody and all levels of dosage were successful in providing an enhancing effect. The Henry and Jerne study clearly demonstrated for the first time, the enhancing effect of pre-existing IgM antibody and the suppressive effect of IgG antibody.

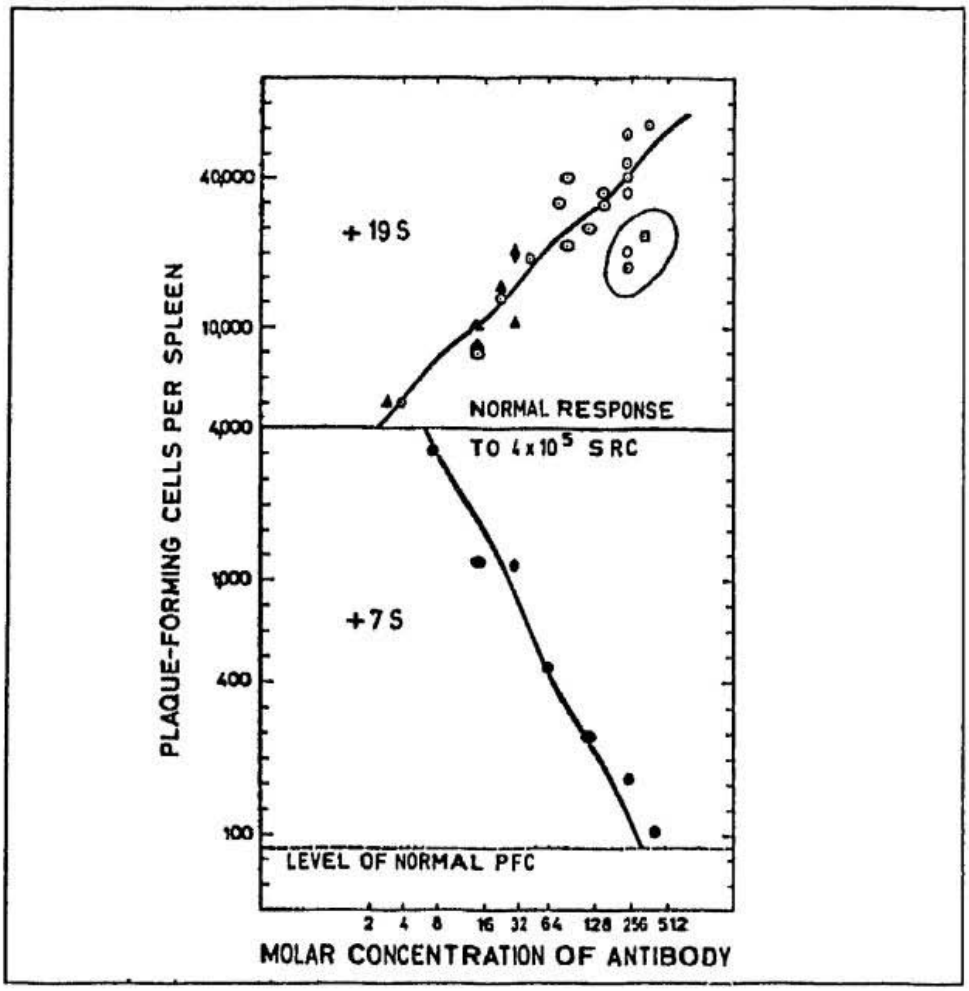

Figure 1 : Elevation by specific 19S antibody, and depression by specific 7S antibody of the primary immune response of mice to a single intravenous injection of 4 X 10^5 SRBC as measured by counting the total number of plaque forming cells per spleen. The open symbols (including the triangles) represent the average responses of groups of mice that received 19S antibody, the solid circles represent the average responses of groups that received 7S antibody. Each point is the mean number of plaque forming cells per spleen of 20-50 mice after deducting the number of background plaque forming cells shown (Henry and Jerne, 1968).

1.3.3 IgM Mediated Enhancement

Current knowledge on the effect of pre-existing IgG and IgM antibody is quite detailed but unfortunately not complete. The exact mechanism and/or mechanisms of these antibodies is still unknown. There is, however, a consensus in the literature that IgM antibody has an enhancing effect on the primary response. In the case of the IgG antibody there is a paradoxical effect; in some incidences the immune response has been shown to be enhanced while in others it has been suppressed.

1.3.3.1 Specificity Characteristics

From the Henry and Jerne (1968) study it was shown that antibodies of the IgM class can have an enhancing effect on the primary immune response. It is now known that whether particulate (Wason,1973) or soluble (Enriques and Klaus,1984) antigens are used, the enhancement by IgM is still observable.

Collisson *et al.* (1983) further investigated the specificity of IgM enhancement by simultaneously injecting two antigens together with the IgM antibody directed only against one of them. Only the response to the antigen against which the IgM was directed was enhanced. This was shown both in an erythrocyte system with ox and human erythrocytes and in a protein system with ova albumin (OA) and 2,4-dinitrophenyl - bovine serum albumin (DNP-BSA). This specificity has been further characterized as being

antigen/particle specific but not epitope specific. Heyman *et al.* (1982) used the fact that SRBC and goat red blood cells (GRBC) cross-react at both the T and B cell levels. After IgM-anti-SRBC monoclonal antibody administration, GRBC or SRBC were used as the immunogen. They then assayed for plaque forming cells against GRBC and SRBC. It was found that the monoclonal antibodies that cross-reacted with GRBC enhanced both the anti-GRBC and the anti-SRBC response. However, monoclonal antibodies used that did not cross-react with GRBC did not give any enhancement when GRBC was the immunogen. These results suggest that the 'IgM antibodies enhance via binding to the erythrocytes, thereby allowing other epitopes on the same erythrocyte to display enhanced immunogenic properties' (Heyman *et al.* 1982). Since these experiments, the effects has been clearly characterized as being antigen/particle specific but not epitope specific.

Using a malaria vaccine, Harte and his group investigated whether specific monoclonal IgM antibodies could be used to enhance the response to a blood-stage murine malaria vaccine. The group found that small amounts of a monoclonal anti-parasite IgM could specifically enhance both primary response and memory cells in response to vaccination. These results were supported by survival after infection. In offspring of the murine mother, where vaccination was ineffective for up to eight weeks due to the presence of maternal IgG, it was possible to overcome the inhibition of the maternal antibodies when the IgM monoclonal was administered before the vaccine. This was described as being made possible by the increased concentration of antigen in the spleen as measured by the injection of radiolabelled vaccine measured 24 hours after injection

in control mice (Harte *et al.* 1983).

1.3.3.2 Basic Criteria

It was Dennert (1971) that first described the localization of the antigen being enhanced in the spleen by pretreatment with specific IgM antibody. Dennert labelled antigen with ^{51}Cr and injected it after IgM antibody administration. After ten minutes the animals were sacrificed and the radioactivity of the spleens assayed. Compared to control animals which received no IgM antibody, there was a ten to twenty fold increase in antigen uptake when animals were treated with the specific IgM antibody. If IgM works by trapping the antigen - antibody complexes then there should also be localization found in other organs, such as the liver. However, the concentrations of antigen in the liver was not enhanced by the pretreatment of IgM antibody (Dennert,1971) This finding may be explained by the fact that the blood content and, thus, the background of antigen in the liver is too high to see further IgM induced concentration of antigen. Dennert tested a variety of time intervals between IgM and antigen administration to determine an optimum schedule. It was seen that administration of antibody one to two hours before antigen injection would result in sufficient enhancement (Dennert,1971). From Henry and Jerne (1968) it was established that stimulator activity of IgM depends on the amount of administered IgM antibody as well as the antigen dose. However, in Dennert's experiment, augmentation by IgM could only be observed when antigen doses were at suboptimal levels (Dennert,1973). This is in contrast to Henry and Jerne's experiment

in which they demonstrated a linear relationship with no evidence for a plateau or levelling off. Thus, from these experiments it was established that certain criteria must be optimized for the enhancement to be observed; specifically, the dose of antibody, and an optimum time frame of antibody administration. Dennert also provided a clue as to the mechanism involved, showing that the antigen localization in the spleen was increased by the enhancing IgM antibody.

1.3.3.3 *In Vivo* versus *In Vitro* Experiments

The *in vivo* investigation of the response to heterologous erythrocytes in the mouse by Henry and Jerne (1968) was also followed up by John Schrader in a series of experiments that examined the effect of specific IgM antibodies on the *in vitro* primary response to sheep erythrocytes by mouse spleen cells. Schrader's claim was that in such a system the amount of antigen and antibody to which a given population of immunocompetent cells is exposed may be controlled with much greater precision than in the *in vivo* situations, where compartmentalization of both antigen and antibody may complicate interpretations at many levels (Schrader,1973). Schrader (1973) in replicating Henry's and Jerne's 1968 experiment, used antibody preparations of IgM shown by Henry and Jerne (1968) to be enhancing; these had marked suppressive properties *in vitro,* while the *in vivo* experiments performed with the same antibody replicated Henry and Jerne's (1968) results. The antibody was prepared exactly as stated in the original experiment. The results demonstrated a paradoxical effect of the *in vivo* and *in vitro* activities of

passively administered IgM.

In 1973 William Wason also investigated the effects of pre-existing antibody in *in vitro* and *in vivo* experimental designs. His experiment compared the effect of antibody given before and after antigen both *in vivo* and *in vitro*. The *in vitro* experiments allowed for "control" over variables such as an accurate quantification of the antibody half life, and elimination of the possibility of the IgM altering antigen distribution at the tissue level or affecting the selection of circulating cells into the spleen. The half life of IgM antibody in the mouse circulation has been reported as one day (Britton *et al.* 1968) whereas the half life *in vitro was* found to be greater than six days (Wason,1973). His design allowed him to test the hypothesis that the IgM antibody caused augmentation of the immune response *in vivo*, by altering antigen distribution and thus increasing the amount of effective immunogen in the spleen. Injected IgM anti-SRBC antibody was found to have a dual effect on the primary immune response in mice. The response to SRBC was either augmented or suppressed depending on whether the IgM antibody was given before or after the immunizing dose of SRBC. In the *in vitro* experiments, IgM anti-SRBC antibody suppressed the response of cultured mouse spleen cells to SRBC under all the conditions that were tried. Therefore, the findings were consistent with the hypothesis that IgM antibody increases the response *in vivo* by altering antigen distribution, a conclusion which had been suggested by Dennert's work (Dennert,1973).

1.3.3.4 T Cell Component

Another characteristic of IgM mediated enhancement is its dependency on T-cells. Dennert, in testing to see if anti-SRBC IgM could substitute for the helper function of T cells, opted to test its action in thymectomized mice. These mice were thymectomized at the age of 3-4 weeks, irradiated and protected with syngeneic fetal liver cells. Experiments were conducted, as before, with SRBC as the antigen. When low or high doses of antigen were administered and the plaque assays performed, there was no detectable stimulation by IgM. Dennert also conducted *in vitro* experiments. When T cell deficient spleen cells were used, and low doses of IgM were given, there was a small (but not significant) improvement of the SRBC response. This IgM enhancement was lower than that found in experiments in which B cells were mixed with sensitized T cells (Dennert, 1973). Collisson *et al.* in 1983 were also interested in the T cell component. In experiments conducted with nude mice which lack functionally active mature T cells, there was no potentiation of the primary response (Collisson *et al.* 1983), confirming the results described earlier by Dennert.

As previously mentioned, non-specific IgM administered prior to the antigen does not produce any enhancement. However, there is one study that showed enhancement of anti-horse red blood cell response by monoclonal anti-SRBC IgM antibody which fails to bind to the horse red blood cells (Heyman *et al.* 1982). It was the belief of Collisson *et al.* that there may exist a cross-reactivity at the T cell level, to explain this type of result.

They chose two proteins that do not cross-react at the T - helper cell level, bovine serum albumin (BSA) and ova albumin (OA), for further experiments. In adoptive transfers of spleen cells from mice injected with 2,4-dinitrophenyl (DNP)-OA alone, BSA alone or a mixture of the two, into sublethally irradiated mice, the effect of treatment with IgM anti-2,4,6-trinitrophenyl (TNP) was studied. Since TNP and DNP cross-react, IgM with TNP specificity was used to modify responses against DNP coupled molecules. A distinct specificity was observed. Only in the case when anti-TNP IgM antibody and DNP-BSA (antigen) were given did the naive mice, which received T cells specific to BSA, show an observable increase in the antibody response to BSA and DNP (remarkably a fourfold enhancement). This experiment also included groups where the effect of anti-hapten antibody on the pure anti-protein response against nonconjugated BSA or OA was studied. No effect was observed in either of these cases (Collisson *et al.* 1983). Based on previous work by this group the authors were able to show the existence of IgM Fc receptors on murine T cells (Lamon *et al.* 1976). Furthermore, removal of this distinct T cell population (IgM-Fc-receptor positive T cells) from a primed T-cell population reduced the helper T cell activity in a passive transfer system (Anderson *et al.* 1979). Janeway *et al.* (1971) have shown that one subpopulation of T helper cells requires the presence of B cells to function. It is possible that such antibody dependent T helper cells function by binding IgM complexes via IgM Fc receptors. These findings support the claim that IgM Fc receptors on helper T cells play a functional role in regulation of antibody responses *in vivo* (Collisson *et al.* 1983).

1.3.3.5 Role of Complement

In 1988 Heyman and colleagues conducted experiments to study the importance of complement factors on the ability of IgM to induce enhancement. In these studies three different approaches were used. In the first, a mutant IgM antibody unable to activate complement was used. In the second, mice genetically deficient in complement were utilized. Finally, mice depleted of complement by treatment with cobra venom factor were produced. In the first approach, two monoclonal IgM-anti-TNP antibodies were used. One of the antibodies contained a point mutation in the μ chain constant region, therefore, could not activate complement. The normal IgM antibody enhanced the response in a dose-dependent fashion, whereas, the mutant IgM antibody did not produce any significant enhancement (Heyman *et al.* 1988). When complement deficient mice were used, it was shown that IgM can produce an enhanced response in C5-deficient AKR mice, thus, demonstrating that at least the late acting complement components, C5-C9, are not critical (Heyman *et al.* 1988). In the third case, mice were treated with cobra venom factor and then injected with polyclonal IgM-anti-SRBC. It was previously shown that *in vivo* doses of cobra venom factor causes transient depletion of plasma C3 to less than 5% of normal levels (Pepys, 1975). After a period of time the mice were then injected with the antigen, SRBC. This treatment abolished the six-fold enhancing effect on the direct plaque forming cell response of IgM seen in control mice immunized the same way. These studies clearly indicate that the enhancing effect of the IgM antibody is dependent upon complement factors, probably those acting before C5.

1.3.3.6 Postulated Mechanisms

Of the postulated mechanisms of IgM enhancement, one hypothesis suggests that antigen-antibody-C3b complexes are trapped in the spleen, possibly on the follicular dendritic cells, which carry complement receptors (Dennert,1971,1973; Heinen *et al.* 1985). Supporting evidence comes from studies showing failure of IgM potentiation when IgM is injected after the antigen indicating that the immune complexes are in some way responsible for the enhancement, in addition to evidence provided by the *in vitro* experiments (Schrader,1973). The trapped immune complexes then can capture B cells carrying antigen-specific receptors and C3b receptors (see Figure 2, page 15). Crosslinked C3b is known to stimulate activated B cells to proliferate and secrete immunoglobulin (Erdei *et al.* 1985). Additional help may be provided by antigen-specific T helper cells that are activated more efficiently due to increased concentrations of antigen and, possibly, by IL-1 secretion by the follicular dendritic cells (Heyman *et al.* 1988; Heyman,1990b).

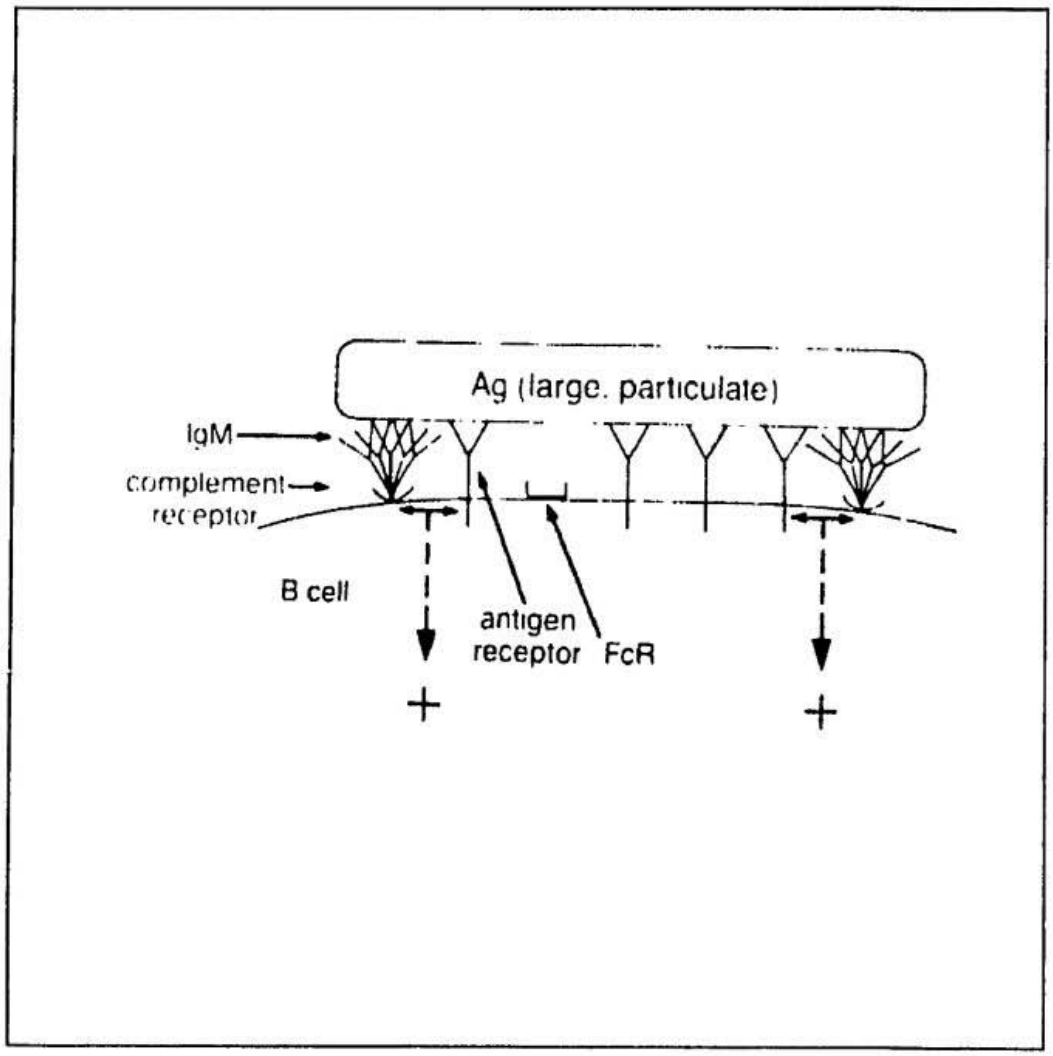

Figure 2 : Theoretical explanation of IgM-mediated enhancement. Hypothesis : IgM - antigen complexes activate complement and stimulate B cells via crosslinking of complement and antigen receptors. In addition, increased antigen localization (via complement receptors) on follicular dendritic cells in germinal centres may play an important role (Heyman, 1990b).

1.3.4 IgG Mediated Suppression

Heyman and Wigzell with the aid of a simple enzyme-linked immunosorbent assay (ELISA), were able to show that mice pre-treated with IgG antibody and then administered antigen showed suppressed levels of both IgG and IgM antibody levels in the primary response (Heyman and Wigzell,1985).

1.3.4.1 Specificity Characteristics

IgG antibodies can specifically suppress the humoral immune response against particulate as well as soluble antigens (Henry and Jerne,1968; Bruggemann and Rajewsky,1982; Heyman and Wigzell, 1984). Studies have shown that the IgG suppression is non-epitope specific but is antigen/particle specific (Bruggemann and Rajewsky, 1982). IgG antibodies binding to one epitope suppress the antibody response to all epitopes of the particular antigen used (Heyman, 1990b).

Wiersma *et al.* (1989) studied the ability of IgG to modulate the immune response against TNP-SRBC and a control antigen, horse red blood cells (HRBC) with the use of a polyclonal antibody and panel of nine IgG TNP-specific monoclonal antibodies. Both antigens (TNP-SRBC and HRBC together) were injected into mice one to two hours after administration of the antibody. The plaque forming cell responses against SRBC and HRBC were then determined. Five of the nine IgG TNP specific monoclonals and the

IgG anti-TNP specific polyclonal antibody were able to suppress the response to SRBC whereas there was no significant suppression observed on the HRBC response (Wiersma *et al.* 1989). Wiersma's group also examined the antigen binding properties of the various antibodies used. The antigen binding capacity was tested in three different assays : binding to SRBC-coupled TNP in an ELISA; ammonium sulphate precipitation by the Farr technique; hemagglutination. It was found that the antibodies that were unable to suppress the response *in vivo* gave significantly weaker binding results with SRBC as compared to the antibodies that were suppressive (Wiersma *et al.* 1989). Thus, suppression is antigen specific, non-epitope specific, and the antigen binding ability (affinity) of the IgG antibody appears to be of importance.

1.3.4.2 Ability of Various Subclasses to Suppress

It has been shown by a number of investigators that all subclasses of IgG can be suppressive (Bruggemann and Rajewsky,1982; Heyman and Wigzell, 1984). Bruggemann's and Rajewsky's main aim was to study the role of antibody class in feedback inhibition. Feedback inhibition has been one of the proposed mechanism of IgG suppression and it implies that the IgG antibody is able to direct a negative signal or able to block the productive phase of the primary response. Binding of antigen-antibody complexes to Fc receptors may play a crucial role since Fc receptors of various isotype specificities have been found on T and B lymphocytes and on macrophages (Unkeless *et al.* 1981). It was Bruggemann's and Rajewsky's belief that these receptors could well

confer isotype specificity to feedback inhibition. They found that suppression was equally achieved with IgG_1, IgG_{2a}, IgG_3 and IgG_{2b}. Thus, they concluded that antibody feedback inhibition is not a function of a particular IgG subclass, and that the antibody needs no property other than its affinity to the antigen (Bruggemann and Rajewsky,1982).

1.3.4.3 Experiments involving $F(ab')_2$ Fragments and the Fc Receptor

As early as 1969, investigators had been curious as to the role of the Fc fragment of antibody in the signalling process of an antibody response. Sinclair was specifically interested in ascertaining whether binding of antibody to antigen, and the masking of antigenic determinants, are sufficient to induce the suppression of the immune response. In Sinclair's experiment he was able to remove the Fc portion of the IgG antibody. In this way the binding of the antibody to antigen was not altered. The resulting $F(ab')_2$ portion was tested for its ability to inhibit the primary immune response. He found that the $F(ab')_2$ portions were 100 to 1000 times less suppressive than complete antibody (Sinclair,1969). These results were later confirmed by others, Heyman (1990) and Gordon and Murgita (1975). Heyman (1989) again investigated the role of the Fc regions of IgG antibodies in IgG mediated suppression using antibodies blocking cellular Fc receptors. Heyman found that when she used these antibodies it reversed the ability of the IgG antibodies to induce suppression (Heyman,1989). From these experiments it may be concluded that interactions between Fc regions and membrane bound, or possibly secreted Fc receptors, are crucial events in IgG mediated suppression.

1.3.4.4 Density Factor

It has been found that the higher the density of IgG antibodies bound to the specific antigen, the more efficient the suppression of the response by the particular IgG antibody (Quintana *et al.* 1987). Two factors involved in the suppression, therefore, are affinity and the number of epitopes recognized by an administered IgG preparation. This may explain why some researchers have found that polyclonal IgG is usually more suppressive than monoclonal IgG (Bruggemann and Rajewsky,1982; Wiersma *et al.* 1989). Some researchers have commented on this, suggesting that there may be a threshold density dividing monoclonal IgG antibodies into those that can suppress and those that do not suppress (Wiersma *et al.* 1989). When mixtures of different monoclonal IgG antibodies are used the suppressive effect of the individual monoclonals is additive (Heyman and Wigzell, 1984). It has been suggested that the link between the suppressive effect and density of IgG on the antigens can be explained by increased binding to Fc receptors by the high-density complexes (Heyman,1990). An interesting experiment to test this would be to see if a divalent antibody (one with two specificities) would give more suppression as compared to a monoclonal antibody.

1.3.4.5 *In Vitro* Experiments

A number of researchers have confirmed that IgG induced suppression is

observable *in vitro*, (Abrahams *et al.* 1973; Kappler *et al.* 1973; Hoffmann *et al.* 1974). In one such experiment spleen cells were cultured with SRBC alone or with SRBC and anti-SRBC IgG antibody; a number of different ratios of antigen to antibody were used. The plaque forming cell kinetics were measured over a number of days. The results clearly showed that cultures containing antigen-antibody complexes given at the beginning of the culture suppressed the appearance of plaque forming cells at all subsequent days of cell culturing (Oberbarnscheidt and Kolsch,1978). The degree of suppression depended directly upon the concentration of antibodies in the complex. These experiments indicate that antibody - antigen complexes *in vitro* can efficiently inhibit the induction of antibody formation.

1.3.4.6 Role of T Cells

Oberbarnscheidt and Kolsch (1978) also examined the role of T cells in IgG mediated suppression with the aid of nude mouse (nu/nu Balb/c) spleen cells in an *in vitro* set of experiments. It was first established that spleen cells from nu/nu Balb/c mice could respond (produce antibodies) to sheep red blood cells or to TNP-SRBC in the presence of supernatant containing thymus-replacing factor obtained from concanavalin A activated normal spleen cells. It was found that this response could be blocked if anti-SRBC IgG antibody was added to the mouse spleen cell culture. The degree of suppression was similar to that found when normal spleen cells were used. With this particular design the researchers were confident that the T cells were not directly involved in suppression, but

the possible effect of a suppressive T cell factor in the supernatant of the concanavalin A activated spleen cells was not included. At this time, concanavalin A activated T cells were considered to be suppressive. To test this further, the effect of antigen-antibody complexes on nu/nu Balb/c spleen cells in the presence of antigen and lipopolysaccharide (LPS), a non-T cell stimulator, was studied. Again it was shown that the cultures containing the antigen-antibody complexes had a significantly suppressed response. The researchers were then concerned with the possibility that anti-Thy-1.2 sensitive immature T cells might mediate antigen-antibody induced suppression. The suppression of the anti-SRBC response in cultures of anti-Thy-1.2 treated normal or nu/nu Balb/c spleen cells supplemented with thymus replacing factor or LPS was then examined. Once again, immune complexes suppressed the immune response showing clearly that the suppression of IgG antibody is not dependent upon the presence of T cells, (Oberbarnscheidt and Kolsch,1978). The data suggests that antibody - antigen complexes directly block B cells (or their access to antigen) in such a manner that they cannot be affected by maturation signals, such as LPS or thymus-replacing factor.

The aim of Hoffman and Kappler (1978) was to investigate the inhibitory action of IgG antibody in relation to the Fc receptor. They found that inhibitory antigen - antibody complexes did not effect the activity of T helper cells or antigen reactive B cells. They took advantage of fact that SRBC and burro red blood cells (BRBC) cross-react at the T-cell level, but, not at the B cell level. SRBC primed helper T cells stimulated formation of antibody to both SRBC and BRBC. They were able to show that non-cross

reacting antibody against SRBC inhibited the production of antibody to SRBC but did not diminish the helper effect of SRBC primed T cells on BRBC reactive B cells. Conversely, TNP-reactive B cells (responding to TNP coupled SRBC and BRBC) failed to produce antibody to TNP-SRBC in the presence of antibody to SRBC, but, remain responsive to TNP-BRBC in such conditions. The results indicate that neither T cells nor B cells were immediate targets of the suppressive antibody. Hoffman and Kappler were then curious to see if the process of cooperation between T and B cells was altered. They tested three types of antibodies to SRBC in experiments investigating the inhibition of a anti-SRBC response in spleen cell cultures : IgG rabbit anti-SRBC, IgG rabbit SRBC-$F(ab)_2$ and chicken anti-SRBC (Fc portion unable to recognize mammalian cells). All three inhibited the production of an anti-SRBC plaque forming cell response at high concentrations. However, only intact rabbit antibody was inhibitory also at a low concentration. The addition of serum containing tumour necrosis factor (previously shown to replace the helper signal and support the production of antibody in identical spleen culture cells depleted of T cells) reversed the suppressive effect at low concentrations but not at high concentrations of rabbit anti-SRBC antibody. The inhibitory effect of increased concentrations of rabbit anti-SRBC-$F(ab)_2$ antibody or of chicken anti-SRBC was not reversed by the "tumour necrosis serum". Thus, these results support the belief that antibody can inhibit the cooperation of T and B cells. This inhibitory effect was described as being "reversible by replacing the signal which results from that cooperation". Thus, the ideas that T helper cell activity is unaffected and that T - B cell cooperation was disturbed by the inhibitory antibody were supported (Hoffman and Kappler,1978).

1.3.4.7 Role of Complement

Heyman *et al.* (1988b) investigated the role of complement in IgG mediated suppression. In one experiment they chose two different IgG anti-TNP antibodies, one that could activate and one that was unable to activate complement. Both antibodies bound equally well to the antigen, sheep red blood cells coupled with TNP. To avoid detecting suppression caused by the blocking of antigenic determinants by immunoglobulins, the researchers chose to measure the anti-SRBC response as opposed to the anti-TNP response. Mice were given the antibody preparation one - two hours prior to administration of antigen and plaque assays were performed on day five. The non-complement activating antibody suppressed up to 94% of the SRBC antibody response. However, this degree of suppression was in the same range as that of the complement activating antibody. These researchers also presented data showing that polyclonal IgG anti-SRBC antibodies were as efficient as suppressors in mice depleted of more than 90% of their serum C3 by treatment with cobra venom factor, as they were in normal mice. The response to sheep red blood cells in AKR mice, genetically C5 deficient, was also efficiently suppressed by IgG antibodies. These data clearly support the belief that complement factors are not involved in IgG mediated suppression of the primary immune response (Heyman *et al.* 1988b).

1.3.4.8 Memory

Heyman and Wigzell (1985) conducted experiments to determine if IgG mediated suppression of the primary response also affected the secondary response, i.e. suppressed the production of memory. Mice were either primed with SRBC alone or with polyclonal IgG anti-SRBC antibodies followed by SRBC, at a dose known to give optimal suppression. This suppression was confirmed one week later by a direct plaque assay using a sample of the animals. After 24-90 days, spleen cells from other primed animals were transferred to irradiated syngeneic hosts together with antigen. The secondary response was measured by direct and indirect plaque assays. Both IgG-SRBC and SRBC primed cells gave a higher response than did unprimed cells. However, compared with groups primed with SRBC alone, the response was greatly suppressed in the IgG treated groups. This experiment led the investigators to conclude that the suppression of the primary antibody response, caused by passively administered IgG, is also extended to the secondary response which showed inhibited induction of memory (Heyman and Wigzell, 1985).

1.3.4.9 Accessory Cell Involvement

B cell involvement has also been reported in the literature. It has long been recognized that derivatives of C3 play a vital role in phagocytosis (Ehlenberger and Nussenzweig,1977) As mentioned before, no decrease in suppression was observed in

animals depleted of complement. Kolsch's group conducted *in vitro* experiments in which macrophages were depleted from the cultures. These macrophage depleted cultures showed the same degree of suppression as non-depleted cultures, (Kolsch *et al.* 1980).

Another supporting piece evidence comes from *in vitro* experiments where the importance of the Fc fragment of rabbit anti-mouse kappa chain antibodies in preventing the activation of murine B cells was studied. Intact rabbit anti-mouse kappa chain antibodies totally failed to induce tritiated thymidine incorporation over a three $\log_{10}$ concentration range. However, $F(ab')_2$ fragments prepared from these same antibodies led to a 'marked' incorporation of the radioactive label. A mixture of the intact anti-kappa antibodies and the $F(ab')_2$ fragments derived therefrom again prevented activation. The researchers then went on to investigate Fc involvement. This idea was studied using goat anti-mouse μ chain specific antibodies which, even when intact, produced significant B cell activation. The stimulatory capacity could be increased at any time tested by using goat $F(ab')_2$ of anti-mouse μ chain specific antibodies. The researchers concluded that the immunoglobulin receptor of murine B cells can serve as a triggering receptor provided that a negative signal mediated by cross-linkage involving the B cells Fc receptor is avoided (Tony and Schimpl,1980).

Accessory cells may also be involved in suppression. "[Increased] ... FcR-mediated phagocytosis of immune complexes may explain the requirement for FcR binding, the antigen specificity and the non-epitope-specificity" (Heyman,1990b). As it

stands, definitive data on the effect of accessory and B cell involvement is still needed, however, the most plausible explanation, as of now, may lie in the combination of the two (Heyman,1990b).

1.3.4.10 Postulated Mechanisms

The literature contains a number of theories on the mechanism of IgG mediated suppression. They can be summarized into three main theories. The first proposes that suppression is the result of simple blocking of antigenic determinants by IgG antibodies. These determinants are then hidden from immunocompetent cells (Hoffman and Kappler,1978; Moller,1985). This mechanism is independent of the Fc component of the IgG antibody and is contradicted by the literature cited in this review which documents the need for the Fc region. The second theory has been termed the 'peripheral theory' (Ryder and Schwartz, 1969) and contends that the elimination of antigen is mediated by increased phagocytosis of the IgG-antigen complexes. However, complement is known to increase phagocytosis of immune complexes (Egwang and Befus, 1984) and the finding that immunosuppression is independent of complement activation, therefore, argues against this theory. The third hypothesis, the 'central theory', proposes that the immune complexes cross-link Fc and antigen receptors on B cells thereby inducing an off signal to the cells (Oberbarnscheidt and Kolsch, 1978; O'Garra *et al.* 1987; Phillips and Parker,1985; Wiersma *et al.* 1989; Heyman, 1990b). The biochemical basis of the result of the crosslinking of Fc and antigen receptors is not completely understood. In many cell

types, binding of ligands to their receptors causes an early increase in free intracellular calcium and a rapid degradation of phosphatidylinositol bisphosphate to inositol trisphosphate and 1,2-diacylglycerol (Berridge,1984). One particular study examined the effect of crosslinking of surface immunoglobulin and Fc receptors and the degradation of phosphatidylinositol bisphosphate and calcium cation concentration, two early biochemical responses in activated B cells. The results indicated that the rapid degradation of phosphatidylinositol bisphosphate was abrogated but the increase in calcium cation concentration remained the same (Bijsterbosch and Klaus, 1985). These findings may explain and/or provide a framework for understanding the mode of action of the Fc regions of the IgG and the Fc receptors in regulating the activation of B cells in concert with their antigen receptors (see Figure 3). It is quite possible, however, that more than one mechanism may be operating.

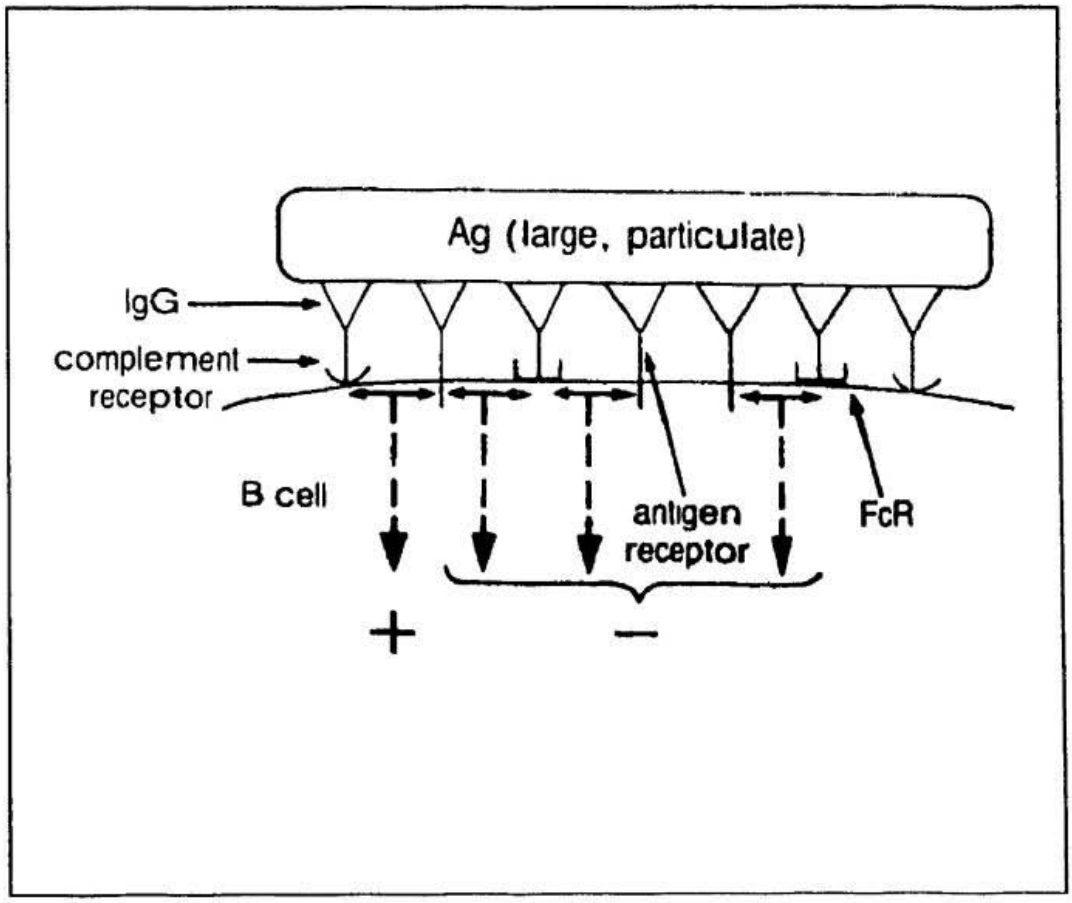

Figure 3 : Theoretical explanation of IgG mediated suppression. Crosslinking of a critical number of Fc receptors and antigen receptors on the B cell results in a negative signal which cannot be reversed by complement receptor binding (Heyman, 1990b).

1.3.5 IgG Mediated Enhancement

Enhancement induced by passively administered IgG is thought to mimic the action of natural IgG antibodies in a normal primary response and/or the action of antibodies produced in a primary response on the secondary immune response (Van Rooijen,1990). The complexity of the events associated with passive antibody administration is further increased by the observation that, under certain circumstances the primary response itself is enhanced rather than suppressed, with pretreatment of IgG antibody (Coulie and Van Snick,1985; Farkus *et al.* 1982; Wiersma et al. 1990; Wiersma,1992). This is in contrast to the previous reports describing experiments in which pretreated IgG mediated suppression. Unfortunately, to date, research and thus the literature do not contain a complete description of this phenomenon. Antigen characteristics such as particulate versus soluble, may play a role, for example, the same TNP-specific IgG monoclonal antibodies that suppressed the antibody response to particulate TNP-SRBC have opposite effects on the response to TNP-KLH, a soluble antigen (Heyman,1990b). Other factors postulated as being important are density of epitopes, and the role of complement.

1.3.5.1 Response to Differing Antigens and Localization

In studying the specific enhancement of the antibody responses to protein antigens by IgG antibodies *in vivo*, Wiersma injected antibody before and after antigen had been cleared from circulation, and attempted to determine if the antibody acts by increasing

antigen uptake (Wiersma, 1992). The response to keyhole limpet haemocyanin (KLH) - TNP antigen was enhanced by a TNP specific IgG monoclonal antibody. High and low concentrations of antibody and antigen were used and all producing an enhanced response. Wiersma then tried other antigens such as BSA-TNP, OA-TNP, TT-TNP and diphtheria toxoid (DT) - TNP in similar experiments. Of these, only the response to BSA-TNP was enhanced by the specific IgG. In order to enhance the response to KLH-TNP and BSA-TNP, the IgG antibody had to be injected while the antigen was circulating in the blood stream. These data suggested that IgG mediated stimulation acts by concentrating circulating antigen into the secondary lymphoid organs (Wiersma, 1992), thereby giving a more efficient presentation of antigen to the B cells.

1.3.5.2 Density

Wiersma (1992) found that IgG antibody caused optimum enhancement when it recognized a low number of epitopes on the administered antigen. Thus, as seen before, density of epitopes also seems to have an impact on the primary response.

1.3.5.3 Role of Complement

The role of complement also was investigated by various researchers. Wiersma *et al.* (1990) examined the importance of complement to IgG mediated enhancement by immunizing mice with TNP-KLH after injecting them either with a complement-activating

TNP-specific monoclonal IgG antibody, or with a variant of this antibody which was non-complement activating. Both the normal and mutant antibodies enhanced the anti-KLH response, although the normal antibody gave a stronger enhancement. Thus, indicating that the classical pathway is likely to be of some importance. In another experiment, a complement-activating IgG antibody enhanced the antibody response in mice depleted of C3 by treatment with cobra venom factor. A naturally occurring IgG non-complement activating TNP-specific antibody, in the same experiment, was also able to enhance the response to TNP-BSA. These findings demonstrate that the ability to activate complement is not an absolute requirement for the ability to enhance (Wiersma *et al.* 1990). It has been earlier shown that the haemolytic capacity of antibodies mainly requires classical pathway C activation, indicating that the classical pathway is the main means by where IgG activates (Bindon *et al.* 1988). The data demonstrating that C-activated IgG is an equally efficient enhancer of the antibody response in normal mice as in mice depleted of their C3, also suggests that the alternative pathway is unlikely to play a role. Although these mice have low traces of C3 in circulation it is not believed that there is enough for completely normal enhancement (Wiersma *et al.* 1990). Thus, from these studies the requirement to activate complement is not essential to the IgG mediated enhancement suggesting that there may be another cooperating factor or factors involved in the enhancement process. Interestingly, these studies do not address C4b, since it also binds C3b receptors and would be activated in the absence of C3.

1.3.5.4 Mechanisms of IgG Mediated Enhancement

Although the investigators who proposed the various mechanisms have demonstrated that complement may not be necessary for enhancement, involvement of complement is still a major part of the speculation. It may be the antigen characteristics, such as size, and the density of epitopes recognized by the IgG antibody that might influence the interaction between the immune complexes, complement receptors and the Fc receptors (Wiersma, 1992). As previously mentioned, evidence to-date implies that IgG suppression acts by cross-linking membrane immunoglobulin and Fc receptor (type two) on the B cell, with a critical size of the antigen needed for obtaining an inhibitory cross-link (Heyman,1990b). Some researchers favour a model of IgG mediated enhancement where IgG-antigen complexes are captured by follicular dendritic cells, which carry FcR as well as complement receptors (Heinen *et al.* 1985; Wiersma *et al.* 1990). Both the ability of the IgG antibody to bind to the FcR, as well as the ability to activate complement are essential properties in this model. This model would also explain why non-complement activating monoclonal IgG antibodies gave slightly less stimulation as compared to complement activating IgG monoclonal antibodies (Carter *et al.* 1988). B cells may also be involved. Enhancement may be in part due to the stimulation of B cells by complement fragments via their complement receptors (Melchers *et al.* 1985). Heyman (1990b) has proposed that the enhancing effect depends on the ability of IgG antibody to bind both FcR and complement receptors (see Figure 4), thus causing efficient localization on follicular dendritic cells, which carry both types of receptors. In addition to this, the complexes could stimulate B cells directly via complement receptors on these cells.

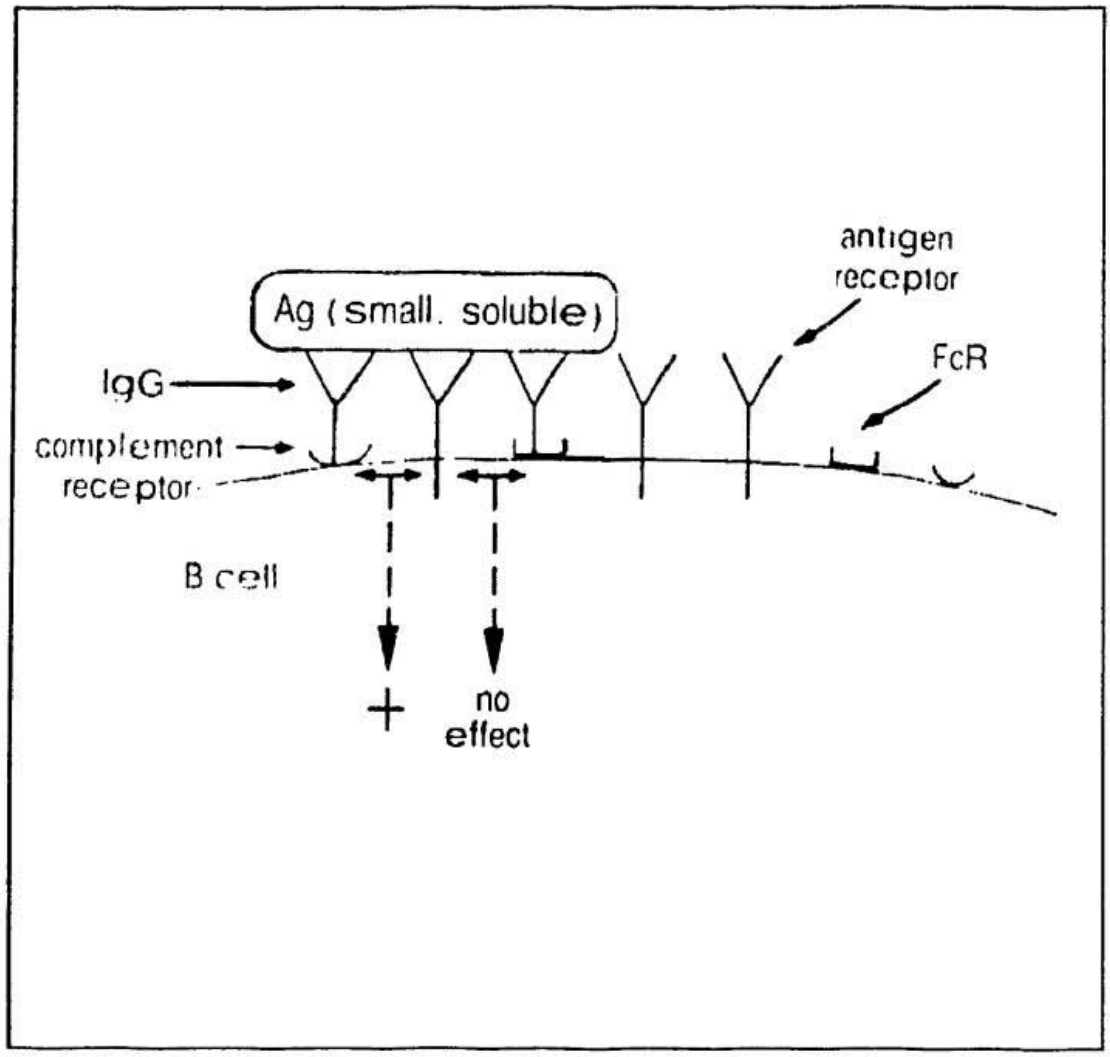

Figure 4 : Theoretical explanation of IgG mediated enhancement. IgG soluble antigen complexes cannot induce the critical number of Fc receptor - antigen receptor crosslinking on the B cell surface to induce a negative signal (Heyman,1990b).

1.3.6 Summary

1.3.6.1 IgM Mediated Enhancement

The enhancing effect on the primary immune response by pretreatment with specific IgM antibody has been well documented. The effect can be demonstrated with both particulate and soluble antigens. With respect to specificity, the response can be described as antigen specific but not epitope specific. A time factor exists, such that administration of specific IgM antibody one to two hours prior to antigen results in optimal enhancement. IgM antibody is to able enhance antigen localization in the spleen. *In vivo* demonstration of enhancement was not replicated *in vitro*. In fact, *in vitro* experiments resulted in a suppression of the primary response. IgM enhancement is further dependent upon T cell help and upon complement factors, specifically those acting before C5. Increased antigen localization on follicular dendritic cells by complement receptors is believed to play a role. The most plausible cellular mechanism is stimulation of B cells via crosslinking of complement and antigen receptors by IgM - antigen complexes.

1.3.6.2 IgG Mediated Suppression

It has been clearly shown that mice pretreated with specific IgG antibody exhibit

suppressed levels of both IgG and IgM antibody levels in the primary response. IgG antibodies can specifically suppress the response against both particulate and soluble antigens. This suppression is non-epitope specific but is antigen specific. IgG antibodies binding to one epitope suppress the response to all epitopes of that same antigen. All subclasses of IgG can be suppressive. Other factors affecting suppression include the affinity of the antibody and the number of epitopes recognized by an administered IgG preparation; increases in either have the effect of a greater suppression. $F(ab')_2$ fragments of specific IgG antibody were found to be significantly less suppressive compared to complete antibody. Thus, Fc receptors are probably required for suppression. *In vitro* experiments have also been able to demonstrate IgG induced suppression. Experiments have also shown that the suppression by IgG is not dependent upon the presence of T cells nor is complement needed. T helper cell activity is unaffected in antibody suppressed mice. Suppression of the primary response is also extended to the secondary response, shown by the inhibited induction of memory. Of the proposed mechanisms for IgG mediated suppression, the central theory seems to fit best with the above findings. This theory contends that immune complexes crosslink the Fc and antigen receptors on the B cell thereby delivering a negative signal to the cell which cannot be reversed or overcome by binding of complement receptors.

1.3.6.3 IgG Mediated Enhancement

The field of modulation of immune responses by pre-existing antibody has been

considerably complicated by the finding, in certain experiments with soluble antigen, that specific IgG can sometimes enhance the response. This contradicts an earlier concept that IgM enhanced, while IgG suppressed the immune response. Although some experiments have demonstrated that complement appears not to be important to IgG-mediated enhancement, paradoxically, the proposed mechanisms of the IgG mediated enhancement view complement as playing a vital role. The most widely accepted mechanism is that this suppression results from the antibody-antigen complexes not being able to induce the threshold number of Fc and antigen receptor crosslinks on the surface of the B cell.

CHAPTER 2

AIMS AND OBJECTIVES

2.1 Research Group Objectives

One overall objective of our research group is to make mouse monoclonal antibodies to polymorphic determinants on HLA-DP molecules. As this is by no means easy or straightforward we have been looking for ways to improve the creation of hybridomas from immunised mice. Among several projects with this aim is the present one, namely to see if antibody enhancement can be used to improve the hybridoma technology and to explore the possibility that pre-existing IgG produced, for exampie, by repeated immunisations might actually reduce the subsequent immune response to class II HLA molecules.

2.2 Specific Thesis Objectives

The particular aims of the experiments, reported in this thesis, were :

1) To determine if the same phenomena are observed when using either human B cells or mouse fibroblasts transfected with HLA-DP, as antigens; namely that a specific IgM

antibody could enhance and conversely, an IgG antibody could suppress, the primary immune response to HLA-DP antigens.

2) To study the feasibility of using IgM enhancement to improve the yield of HLA-DP and HLA-DR specific hybridomas from mice by decreasing the time necessary to produce hybridomas or by increasing the number of hybridomas specific for HLA-DP and HLA-DR.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

Balb/c and C3H mice obtained from the breeding facilities of Charles River Canada, Inc. (188 Lasalle, St.-Constant, Quebec, Canada) and F1 hybrid mice resulting from a cross between Balb/c males and C3H females were used. The F1 hybrid mice were bred by the Animal Care Unit personnel at the Health Sciences Centre, Memorial University of Newfoundland. All animals were approximately 6-8 week old females unless otherwise stated. The mice were housed in standard clear plastic cages and allowed food and water at will. Animals were kept at the Animal Care facility in the Health Sciences Centre.

3.2 Antibodies

The two primary monoclonal antibodies used to manipulate the pre-existing antibody levels were NFLD.M65C3 and NFLD.M68C11C8. Monoclonal DP139BD9 was used as a IgM non-specific antibody. NFLD.M67 was used in the assays as a positive control.

Table 1 : Summary of Monoclonal Antibodies Used With Respect to Class and Specificity

ANTIBODY	ISOTYPE	SPECIFICITY
NFLD.M65C3	IgM	HLA-DP, B cell lines - reacts with an epitope found on all DP types, polymorphic with transfected fibroblasts
NFLD.M68C11C8	IgG_{2b}	HLA-DP, B cell lines - reacts with an epitope found on all DP types and transfected fibroblast cell lines
DP139BD9	IgM	Mouse antibody, does not bind to human HLA
NFLD.M67	IgG_1	HLA-DP, B cell lines - reacts with an epitope found on all DP types and transfected fibroblasts

Monoclonal antibodies were secreted by the hybrid cells into Dulbecco's Modified Eagle Medium (DMEM) containing, 10% heat inactivated Fetal Bovine Serum (FBS) supplemented with 1% sodium pyruvate, 1% L-Glutamine, 1% Penicillin and

Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada). All hybrid cell lines were grown at 37°C, in a 5% Co_2 incubator. These monoclonals were obtained from, and have been examined for their respective specificities previously in this laboratory.

3.3 Antibody Purification

IgM and IgG hybridoma cell lines were allowed to overgrow in appropriate media for 3-4 weeks. The antibody containing media were first centrifuged at 3400 rpm for 30 minutes and then concentrated at 4°C with an Amicon Ultrafiltration Cell, Model 402 (Amicon Inc, USA).

3.3.1 IgM Purification

In the case of the IgM antibodies, the concentrated antibody containing culture supernatant was applied to an Anti-Mouse IgM (μ chain specific) - Agarose column and purified accordingly (Sigma Chemical Company, USA). The column was equilibrated in 0.01M sodium phosphate buffer, pH 7.2 containing 0.5M sodium chloride. The supernatant was applied slowly and re-cycled at least three times. The column was stripped by washing with 0.1M glycine, 0.15M sodium chloride, pH 2.4. One millilitre fractions were collected in 1ml of Tris-HCL buffer, pH 9.0, on ice; this procedure immediately re-adjusts the collected fractions to a neutral pH. The Bio-Rad Econo

System, Model ES-1 (Bio-Rad, USA) was used in order to monitor column washing and the eluted fractions for protein. Fractions were then pooled and measured for protein by a Du Series 60 Beckman Spectrophotometer, Nucleic Acid Soft-Pac Module (Beckman Instruments, USA). The sample was then dialysed using SPECTRA/POR Molecularporous Membrane Tubing 2 (molecular weight cut off : 12000-14000) (Spectrum,USA) against 0.15M PBS at 4°C for 24 hours, with frequent changes of buffer. The samples were again measured for protein as before and concentrated with a Centriprep-10 Concentrator (Amicon Inc., USA), filtered sterile with a 22μm Millipore filter unit (Millex-GS, Millipore, USA) and again measured for protein. The purified antibody was stored sterile at 4°C. After eluting, the column was re-equilibrated as before and stored with 0.05% sodium azide in an equilibrating buffer, at 4°C.

3.3.2 IgG Purification

The IgG antibodies were purified with the use of the Affi-Gel Protein A MAPS II Kit (Bio-Rad, USA). Binding buffer and elution buffers were prepared using Affi-Gel Protein A MAPS II Binding Buffer and Affi-Gel Protein A MAPS II Elution Buffer (Bio-Rad, USA). The sample was applied to the column, diluted one to one with Binding Buffer and re-cycled at least 3 times. After elution of the antibody, the column was washed with Affi-Gel Protein A MAPS II Regeneration Buffer and stored in 0.05% sodium azide in 0.15M PBS at 4°C. The Bio-Rad Econo System, Model ES-1 (Bio-Rad, USA) was used in order to monitor the eluted fractions for protein and to ensure the

column washing was complete. Again 1ml samples were collected in 1 ml of Tris-HCL buffer, pH 9.0, on ice. The protein containing fractions were pooled and measured for protein by a Du Series 60 Beckman Spectrophotometer, Nucleic Acid Soft-Pac Module (Beckman Instruments, USA). The samples were then dialysed using SPECTRA/POR Molecularporous Membrane Tubing 2 (molecular weight cut off : 12000-14000) (Spectrum,USA) in 0.15M PBS over 24 hours with frequent changes of buffer. The protein content again was measured before and after concentrating the sample via Centriprep-10 Concentrator (Amicon Inc., USA). The sample was then filtered sterile with a 22μm Millepore (Millex-Gs, Millepore, USA) filter unit and measured for protein. The sterile purified antibody was then stored at 4^0C. (See Introduction Section 1.3.4.2 Ability of various subclasses to suppress.)

3.4 SDS-Gel Electrophoresis

Purified samples of IgM and IgG antibodies were examined and compared through SDS-PAGE in a vertical slab-mini-gel apparatus. Specifically the Bio-Rad Mini-Protean II and the Bio-Rad Model 200/2.0 Power Supply were used for all gels. The gels were run at constant voltage 165V.

The buffers used for electrophoresis were as follows :

For the running Gel : 1.5M Tris, pH 8.8

For the stacking Gel : 1.0M Tris, pH 6.8

2x Sample Buffer :

0.12M Tris pH 6.8
4% SDS
20% Glycerol
Three drops of a 0.01% Bromophenol blue solution
10% 2-Mercaptoethanol

The gels used for electrophoresis were as follows ::

12% Running Gel :
dist. water 3.3 mls
30% Bis-acrylamide 4.0 mls
1.5M Tris (pH8.8) 2.5 mls
10% Ammonium persulfate 100 μl
10% SDS 100μl
Temed (N, N, N' - tetramethylethylenediamine) 4.0 μl

Butanol saturated water was used to layer the top during the setting time (30 minutes) of the running gel. This was made by mixing 50 ml of butanol with 5 ml of distilled water. This allowed a very distinct interface to develop.

Stacking Gel :

dist. water 3.4 mls
30% Bis-acrylamide 0.83 ml
1.0M Tris (pH6.8) 0.63 ml
10% SDS 0.05 ml
10% Ammonium persulfate 0.05 ml
Temed 5.0 μl

This gel was allowed to set for 20 minutes.

Protein Stains :

Protein was stained with Coomassie Brilliant Blue. This stain consisted of :

0.8g Coomassie Blue (Brilliant Blue R)
1000 mls Methanol
140 mls Glacial acetic acid
This was then made up to 2 litres with distilled water, filter.

The destain consisted of :

600 mls Methanol
140 mls Glacial acetic acid
1260 mls distilled water

After pouring the running gel and stacking gel, the samples were mixed with the sample buffer and boiled for 5 minutes. The samples were loaded to the appropriate wells (such that 5μg of protein of the sample was added per lane) and the gel was run. The gel was then removed from the Bio-Rad Mini-Protean II apparatus and placed in a shallow dish for staining. Gels were stained for at least 3 hours and then destained until most of the background stain of the gel was removed. For all gels a molecular weight marker containing hen egg white lysozyme 14400 kD, soybean trypsin inhibitor 21500 kD, bovine carbonic anhydrase 31000 kD, hen egg white ovalbumin 42699 kD, BSA 66200 kD and rabbit muscle phosphorylase b 97400 kD was used (Bio Rad, SDS-PAGE, Molecular Weight Standards - Low, Bio-Rad Laboratories, USA).

3.5 Antigens for the *In Vivo* Experiments

Human B cells used were the RAJI cell line and the cell line 2.4.93. The Raji cell line was obtained from the 9th International Histocompatibility Workshop. The 2.4.93 cell

line was obtained from Dr. Tom Cotner, University of Washington, School of Medicine, Seattle, Washington, USA. 2.4.93 has a single wild copy of the L2P-1gene and a single copy of HLA-DPβ1*0401 and HLA-DPα1*0101. Both these lines were maintained in RPMI 1640 supplemented with 10% FBS (heat inactivated), 1% Sodium Pyruvate, 1% L-Glutamine, 1% Penicillin and Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada).

A mouse fibroblast transfected with Human HLA-DP genes were used, specifically L25.4. The L25.4 cell line was obtained from Robert Karr, Department of Immunology and Infectious Diseases, Monsanto Co./AA4C, St. Louis, Missouri, USA. The 11th International Histocompatibility Workshop number of this cell line is 8305. The cell line was maintained in DMEM supplemented with 10% FBS (heat inactivated), 1% Sodium Pyruvate, 1% L-Glutamine, 1% Penicillin and Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada).

The B Cell and the transfected mouse fibroblast lines were maintained at 37^{0}C in a 5% CO_2 incubator.

3.5.1 HLA Typing of Raji Cell Line

HLA typing of Class II; HLA-DR,DQ and HLA Class I; A,B,C was performed in separate 72 well, polystyrene, non-sterile, mini trays with lids (Nunc. Intermed Nunc.,

Inc., USA). These complement dependent cytotoxic (CDC) typing trays were supplied by the Canadian Red Cross Society (National Office, HLA Section, Manufacturing and Development, 1800 Alta Vista Drive, Ottawa, Ontario, Canada, K1G 4J5). HLA typing of Class II; HLA-DP was typed with the aid of a panel of monoclonal antibodies produced in this laboratory that were specific for known epitopes on HLA-DP. This panel consisted of NFLD.M58, NFLD.M60, NFLD.M66, NFLD.M73, NFLD.M77, NFLD.M101, NFLD.M106, NFLD.M111, NFLD.M112, NFLD.M114, NFLD.M115, NFLD.M116, NFLD.M117, NFLD.M118, NFLD.M119, NFLD.M120, GGPM and MYE901. These antibodies were tested against the Raji cell line by a live cell ELISA (CELISA) and flow cytometry.

Typing with the CDC plates was performed as follows :

1) 24 hours prior to testing, the RAJI cell line was fed, maintaining a cell concentration of 3×10^5 cells/ml. On the day of testing, the cells were counted using a haemocytometer. A total of approximately 3×10^3 cells/well was used. The cells were then centrifuged at 1500 RPM for 10 minutes at room temperature, washed with RPMI 1640 supplemented with 10% FBS (heat inactivated), 1% Sodium Pyruvate, 1% L-Glutamine, 1% Penicillin and Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada), and resuspended in the correct volume of the same medium.

2) With the aid of a Lambda Jet (Model LJ-60/72, One Lambda, Los Angeles, USA) 1μl of the cell suspension was added to each well, such that the required 3×10^3 cells were

dispensed per well.

3) The plates were incubated at room temperature for 1 hour.

4) Complement was then added. Complement used was Low-Tox-H Rabbit complement, stored at -70°C and thawed on ice (Cedar Lane Laboratories Limited, Hornby, Ontario, Canada). 300 μl of complement per plate was required (5 μl of complement/well). This Complement was used at a dilution of 1 in 3 : 2 mls of complement + 4 mls of diluent. Diluent consisted of 2 mls of RPMI and 2 mls of heat inactivated fetal bovine serum (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada).

5) Plates were incubated at room temperature for 1 hour.

6) 5μl of Eosin stain (Sigma) was added.

7) The cells were fixed in 5μl of (filtered) Formalin.

8) Plates were allowed to incubate in the fridge for 1-2 hours.

9) A cover slip was placed over the entire plate and then read/scored with aid of Lambda Scan Plus II (One Lambda, Los Angeles, USA).

3.6 Administration of Antibody and Antigen

Antibody and antigen were both given intravenously via the tail veins. The animals were placed in a warm room (22°C - 25°C) for approximately 30 minutes prior to any manipulation. The mice were then lightly anesthetized with ether and placed in a mouse restrainer. To dilate the veins further, warm water was then run over the tail. The correct concentration and volume of antibody or antigen was then administered

intravenously.

Prior to injection, cells were washed 4 times in sterile 0.15M PBS at 500 g, for 10 minutes at approximately 20-25°C. Total resuspended volumes varied from 500-700μl. The cells were injected immediately after washing. In some cases 100μl of a Heparin-Sodium injection (Allen and Hanburys, AGlaxo Canada Limited Company, Canada) was administered intraperitoneally, 15-30 minutes prior to the antigen . This was done with mice receiving (or in excess of) 3.5 x 10^6 fibroblast cells. The heparin relieved the degree and the effects of clumping *in* vivo that the fibroblasts caused when given in large amounts.

3.7 Blood Collection

The animals were placed in a warm room (23°C - 27°C) for approximately 30 minutes prior to any manipulation. To obtain blood, the mouse was first lightly anaesthetized with ether, and placed in a plastic mouse restrainer. The tip of the tail then was cut and approximately 100μl of blood were collected into capped polystyrene microcentrifuge tubes.

3.8 Serum Collection and Storage

Blood collected from the mice was first allowed to clot, approximately 30 minutes,

at room temperature, and then centrifuged for 30 seconds in a Beckman Microfuge B, 11,000 g. The serum then was transferred into capped polystyrene microcentrifuge tubes and frozen for storage.

3.9 Measurement of Antibody Levels

To measure the titre of antibodies to HLA-DP molecules in mouse sera, the Cellular Enzyme Linked Immunosorbant Assay (CELISA) was used. The mouse serum was added to wells of a microtitre plate and allowed to bind to the selected cells. Next an enzyme linked anti-immunoglobulin was reacted with the mouse antibody-target complex. This enzyme-linked anti-immunoglobulin was detected by addition of OPD and substrate. The degree of colour change that results is in direct proportion to the amount of specific antibody contained in the serum. This was quantified by the use of a spectrophotometer.

3.9.1 Modified Procedure for the CELISA involving Human B Cells

This methodology was previously developed in this laboratory prior to my experiments and has been slightly modified to fit the experiments outlined in this thesis. For this assay flexible Microtiter Plates U-bottom 96-well Poly vinyl chloride microplates (PVC) and Limbro/Titertek microtitration 96 flat bottom multi-well plates were used. The assay was carried out with the following reagents and in the following manner:

Reagents:

1) 0.15 M phosphate buffered saline, PBS, pH 7.4.

2) A. 2% Bovine serum albumin (BSA), (Sigma) in 0.15M PBS.

B. 0.5% BSA in 0.15M PBS

3) 1mg of Poly-L-Lysine (PLL), (Sigma) in 100ml of PBS and stored at 4°C.

4) Conjugate (2nd antibody)

A. Fragments of peroxidase-labelled affinity purified (Fab'_2) goat anti-mouse immunoglobulin (heavy and light chains), obtained from the Jackson Immuno-Research (USA) were used. The optimum dilution of the conjugate was predetermined and a dilution of 1/2500 in 2% BSA-PBS was used.

B. Horseradish peroxidase (HRP) - labelled goat anti-mouse IgM (heavy chain specific), (Southern Biotechnology Associates, Inc., USA) used at a 1 in 2000 dilution, as previously determined.

C. Horseradish peroxidase (HRP) - labelled goat anti-mouse IgG (heavy chain specific), (Southern Biotechnology Associates, Inc., USA) used at a 1 in 2000 dilution, as previously determined.

5) Phosphate citrate buffer, pH 5.0

A. 0.1M Citric acid

B. 0.2M Sodium phosphate

This buffer consisted of 4.9 ml of the Citric Acid, 5.1 ml of the Phosphate and 10 ml distilled water, pH was checked to be approximately 5.1. If less than 5 the buffer was

adjusted with phosphate and if greater than 5.1 it was adjusted with citric acid.

6) Substrate

Fresh substrate was made prior to use. For each plate 10 ml of substrate was required. The substrate consisted of 4 mg of orthophenylenediamine (OPD) (Sigma) dissolved in 10 ml phosphate citrate buffer. When completely dissolved 4 μl of 30% H_2O_2 was added.

7) 2.5N H_2SO_4

Target cells :

1) 2.5 x 10^4 cells/well (2.5 x 10^6 cells/plate) were used at 90% viability and in log growth phase.

2) The cells were centrifuged for 10 minutes at 400 g and washed once in 0.5% BSA-PBS.

The procedure for the CELISA involving Human B Cells is as follows:

1) The peripheral wells of the plates were not used and duplicate wells were used for all sera tested. The sera were diluted in doubling dilutions from 1 in 50 to 1 in 1600. Blanks were run on each plate consisting of 10% FBS, as were appropriate positive and negative controls.

2) 10 μl target cells/well to PCV plates were added.

3) 10 μl/well diluted antibody or supernatant were added and then mixed.

4) The cells were then incubated for 1 hour at room temperature.

5) 100 μl/well of 0.5% BSA was then added and the cells centrifuged at approximately 200 g for 5 minutes at 8°C.

6) The supernatant was then flicked off and the plates drained on paper towel after which the cells were resuspended.

7) The cells were washed two more times with 150 μl 0.5% BSA-PBS/well and centrifuged as before.

8) 50 μl/well of conjugate was added.

9) The plates were allowed to incubate 1 hour at room temperature.

10) Limbro/Titertek microtitration plates were coated with 75 μl PLL for at least 1/2 hour and flicked off.

11) The PVC plates were washed four times as before. For the first wash 75 μl of wash solution was added and the plates spun.

12) The cells were resuspended. The U-bottom PCV plates were snapped onto Limbro/Titertek microtitration plates.

13) 100 μl of PBS was then added. Holes were punched in the centre of each well using an 18 gauge needle.

14) The plates were centrifuged at 200 g for 7 minutes.

15) The U-bottom plates were removed and the supernatant flicked off the Limbro/Titertek microtitration plate.

16) 100 μl/well of substrate was added.

17) The plates were incubated in the dark for 1/2 hour at room temperature.

18) 50 μl of H_2SO_4/well was added.

19) The plates were then read in a Multiscan Spectrophotometer (Bio-Rad Model 3550 Microplate Reader).

3.9.2 Procedure for CELISA involving Fibroblasts

A modification of the afore mentioned CELISA was used to detect the relative amount of antibody in serum when using the transfected fibroblasts as the antigen. This procedure takes advantage of the "stickiness" of the fibroblast cells.

Reagents :

1) 0.15M PBS as described earlier.

2) 0.5% & 2% BSA as described earlier.

3) 0.05% Tween-20 (BDH Inc., Canada) in 0.15M PBS.

4) Conjugate as described earlier.

5) Phosphate-Citrate Buffer as described earlier.

6) Substrate as described earlier.

7) 2.5N H_2SO_4

Target Cells :

1) Were greater than 90% viable and in log phase.

2) 5.0×10^4 cells/well in 100 μl/well of 10% FBS DMEM were used.

The procedure for the fibroblast CELISA is as follows :

1) The peripheral wells of the 96 PCV plates were not used. Protocols for all plates prior to the experiment were made. The sera were diluted in doubling dilutions from 1 in 50 to 1 in 1600. Blanks were run on each plate consisting of 10% FBS, as were appropriate positive and negative controls.

2) The transfectants were plated in sterile, 96 multi-well flat-bottom culture plates and left overnight at 37^{0}C in a 5% CO_2 incubator.

3) The plates were then flicked.

4) Washed once with 0.05% PBS-Tween. Added 25μl/well of the appropriate antibody or serum.

5) Incubated at room temperature for approximately 1 hour.

6) Flicked plate and washed four times as before. Added 50 μl/well mouse conjugate.

7) Flicked plate and washed four times. Added 100 μl/well substrate. Incubated 30 minutes in the dark.

8) Added 50 μl/well 2.5N H_2SO_4.

9) Plates were read in a Multiscan Spectrophotometer (Bio-Rad Model 3550 Microplate Reader).

3.10 Flow Cytometry

For these experiments a Becton Dickinson FACStar^{plus} flow cytometer was used to analyze all samples.

Reagents :

1) Azide Buffer (FACS Buffer)

0.01% sodium azide & 0.001% FBS (heat inactivated), (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada) in 1L of 0.15M PBS. This buffer was kept at approximately 4^0C at all times.

2) Fluorescent Antibody

A. R-Phycoerthrin-Affini Pure $F(ab')_2$ fragment goat anti-mouse IgM, μ chain specific with minimal cross-reaction to human, bovine and horse serum proteins, (Jackson Immuno Research, Laboratories, Inc., USA). The optimum dilution was predetermined and a dilution of 1 in 20 was used.

B. R-Phycoerthrin conjugated Affini Pure $F(ab')_2$ fragment goat anti-mouse IgG, Fc fragment specific with minimal cross-reaction to human, bovine and horse serum proteins, (Jackson Immuno Research, Laboratories, Inc., USA). The optimum dilution was predetermined and a dilution of 1 in 20 was used.

3) 1% Paraformaldehyde in 0.15M PBS

Target Cells :

1) Were used at least 2.5 x 10^5 cells/tube, at 90% viable and in log growth phase.

The procedure for FACS analysis is as follows :

1) The correct number of cells were placed in separate polystyrene culture tubes.

2) The cells were washed once with 1ml/tube of Azide Buffer and centrifuged for 5

minutes at 300 g, at approximately 8°C.

3) The supernatant was then poured off and the pellet resuspended.

4) 20μl of test antibody was added. Making sure that the antibody concentration was 30-40 μg/ml. The cells were then placed at 4°C for 30-40 minutes.

5) The cells were then washed 2 times as before.

6) 20 μl of fluorescent antibody was then added to the cells.

7) The cells were then placed at 4°C (in the dark) for 30 minutes.

8) The cells were then washed 2 times as before.

9) The pellet was the resuspended in 150 μl of 1% Paraformaldehyde-0.15M PBS.

10) Florescence was measured using a Becton Dickinson FACStar^{plus}.

3.11 Creation of Hybridomas

These methods were previously developed in this laboratory prior to my experiments and have been modified for the experiments outlined in this thesis. For all fusion experiments F1 hybrid mice were used.

3.11.1 Preparation of Reagents and Media

A. Alkaline Media

To 50 ml of DMEM 1 drop of 5N NAOH was added. The pH was checked and adjusted, if necessary, to 7.8 - 8.0, filtered sterile with a 22 μm Millepore filter unit (Millex-

GS,Millepore, USA), and placed at 37°C.

B. Polyethylene Glycol (PEG)

1 gram of PEG was placed in small capped glass vial and set in a beaker with water in the bottom and autoclaved. 1 ml of the Alkaline Media was then added per vial, resulting in a final concentration of 50% wt./vol. PEG. Samples were stored at 37°C. An extra vial of PEG was always prepared for every fusion.

C. Hypoxanthine, Aminopterin and Thymidine (HAT) Medium

A stock solution of 100X HAT was prepared in bulk, aliquoted and stored at -20°C. The 100X HAT was prepared by dissolving 136 mg of hypoxanthine (Sigma), 38 mg of thymidine (Sigma) and 1.76 mg of aminopterin (Sigma) in 80 mls of sterile H_2O, adding a few drops of 5N NaOH to aid in dissolving. The solution was titrated with 1N HCl to pH 7.0, filtered sterile with a 22 μm Millipore filter unit (Millex-GS, Millipore, USA), and frozen in appropriate aliquots.

D. HT Media

This medium was prepared exactly the same as for the HAT media with the exclusion of the aminopterin.

3.11.2 Preparing Spleens for Fusions

1) Mice were injected with the appropriate amount of purified NFLD.M65C3 intravenously.

2) Following an incubation period of 1-2 hours the antigen, Raji cell, was also injected intravenously.

3) The animals were allowed to remain for 3 days.

4) The mice were then sacrificed by cervical dislocation and the spleens removed aseptically.

5) The spleens were placed in a sterile, polystyrene, disposable, 100x20mm style tissue culture dish (Polar Plastics LTD., Quebec, Canada) containing 10 mls of serum free DMEM medium containing 1% Penicillin and Streptomycin (P/S). The spleens were teased apart using bent, sterile 21 gauge needles 1 1/2 long (PrecisionGlide, Becton Dickinson, USA) mounted on disposable, sterile 3cc syringes (Becton Dickinson and Company, USA) until most of the cells were released. Breaking up any remaining clumps of remaining cells was accomplished by gently pipetting back and forth the spleen cell suspension within the culture dish.

6) The suspension of cells was then transferred to a sterile centrifuge tube. The tissue culture dish was rinsed with 3-5 mls of media, adding this to the centrifuge tube.

7) The suspension was allowed to stand for 3-5 minutes permitting the larger pieces to settle to the bottom.

8) The supernatant was then pipetted off from the sediment and placed in another centrifuge tube.

9) Spleen cells were counted by a making a 1/10 dilution with 3% acetic acid, using a

haemocytometer.

10) The spleens within the same treatment groups were then pooled.

11) The pooled spleen suspension was then washed once in P/S DMEM.

3.11.3 T Cell Depletion of the Spleen Suspension

1) T cells were depleted by using antibody coated magnetic beads, Dynabeads M-450 coated with sheep anti-rat IgG (Product No. 110.08, Dynal, Dynal Inc., U.S.A). The beads were additionally coated at this laboratory with TIB104, a rat anti-mouse T cell antibody. The TIB104 cell line was obtained from American Type Culture Collection (Rockville, U.S.A). A ratio of spleen cells to metallic beads of 1 : 3 was used. The beads and cells were added together in 5% FBS DMEM media, total volume of 6-7 mls in 15 mls sterile centrifuge tubes.

2) The cell-metallic bead mixture was then rotated on an Adams Nutator (Becton Dickinson and Company, U.S.A) for 15 minutes at room temperature.

3) The centrifuge tubes were then placed in a magnetic holder, Dynal MPC-1 Magnetic Particle Concentrator (Product No.12001, Dynal, Dynal Inc., U.S.A) for 1-2 minutes after which the T cell depleted cell suspension was decanted off into a centrifuge tube, whilst holding the 15 mls tube in the magnetic holder.

4) The metallic beads were washed with 10 mls of serum-free DMEM medium containing P/S and the remaining unbound population of cells again added to the existing collection.

5) The T cell depleted samples were then washed with P/S DMEM by centrifuging for 8 minutes at 1500 RPM.

6) Cells were then counted as before.

3.11.4 Preparation of Myeloma Cells for Fusion

The myeloma cell line used for all fusions was Sp2/0 obtained from Robert Weaver, Vancouver. Cells were grown in RPMI 1640 supplemented with 10% FBS (heat inactivated), 1% Sodium Pyruvate, 1% L-Glutamine, 1% Penicillin and Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada). The cell line is unable to secrete immunoglobulin and lacks the hypoxanthine guanine phosphoribosyltransferase enzyme. The Sp2/0's were maintained very carefully with the cells having greater than 90% viability and in log phase. The number of Sp2/0's for the fusions was calculated as being 1 Sp2/0 cell : 10 spleen cells to be fused.

1) The correct number of Sp2/0's were then placed in centrifuge tubes and spun at 500 g for 10 minutes.

2) Both the spleen cells and the Sp2/0's were washed 2 times in serum free medium, DMEM containing P/S.

3) After the second wash the Sp2/0's and the spleen cells were pooled (1:10 ratio) and washed 2 more times with serum free DMEM P/S.

4) The supernatant was then poured off and the centrifuge tube drained on sterile gauze.

At this point the cells were ready to be fused.

3.11.5 Fusion by Pipetting with Polyethylene Glycol

1) Aseptically, 1 ml of 50% wt./vol. PEG was added via a pipette over 2 minutes, directly to the pellet of cells while constantly rotating the centrifuge tube by hand.

2) 2 mls of alkaline medium were added over another 2 minutes as before.

3) 8 mls of alkaline medium were added over 2-3 minutes. The total time for steps 1,2 & 3, was between 6 and 7 minutes and these times were deemed critical.

4) The cells were centrifuged at 200 g for 6 minutes.

5) The cells were washed by adding 10 mls of serum free medium DMEM containing P/S, without mixing up the cell pellet. The preparation was centrifuged as before.

6) Cells were then ready to be plated.

3.11.6 Cell Plating

A. Feeder mice spleens

Spleens from C3H mice were used for all fusions. The spleens were prepared as described in Section 3.11.2. The spleens were then placed in a sterile centrifuge tube and irradiated at 800 RAD. Irradiation was performed by the Radiology Dept., Health Sciences Centre, General Hospital. The feeder mice spleens were used at 1 x 10^5

cells/well.

B. Feeder Red Blood Cells

Feeder red blood cells were obtained aseptically from C3H mice. The red blood cells were placed in 10 mls of 10% Fetal Bovine Serum DMEM supplemented with 1% sodium pyruvate, 1% L-Glutamine, 1% Penicillin and Streptomycin, (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada). The medium was first used to rinse a Vacutainer, brand 100x16mm sterile tube for chemistry and selective haematology determinants containing 143 USP units of sodium heparin (Becton Dickinson, New Jersey, 07070). The medium containing the sodium heparin was then placed in a 15 ml centrifuge tube. Red blood cells were then counted as before and used at 2×10^6 cells/well.

C. Plating of Fused Cells

1) The fused B cells were then plated at 1 cell per well in 100 μl of HAT medium into sterile 96 flat bottom micro-well plates with lids (Nunclon, Inter Med, Nunc, Denmark). A density of 1.3×10^5 spleen cells/well fusion procedure has been established in this laboratory and this number has been found to give clonal growth of fused cells.

2) The plates were then incubated at 37^0 C in a 10% CO_2 incubator and the hybrids allowed to grow.

3.11.7 Hybridoma Feeding

1) Two days after plating, all plates were fed with 100 μl of 2X HAT selection medium.

2) On Day 4, 100 μl of medium were removed from all wells and 100 μl of 1X HAT selection media were added.

3) The hybrids were then fed every 2-3 days with 100 μl of 1X HAT, as in step 2.

4) The hybrids were continuously monitored for growth and, usually on day 10, the hybrid supernatant was rich enough to be used for testing. Usually at this point the hybridomas had confluent growth in their respective wells.

3.11.8 Screening of Hybridomas

3.11.8.A Primary Screening

Primary screening was performed by CELISA. All wells (fused B cells) plated were screened. The supernatants of the various hybrids were tested against the immunizing cell, RAJI. The only modification to the aforementioned CELISA being that 25 μl of supernatant were incubated with 2.5 x 10^6 cells/plate of RAJI cells in 25 μl of 0.5% BSA/PBS. Hybrids that gave an optical density (O.D) greater than 1.0 were kept for further screening.

3.11.8.B Secondary Screening

The following homozygous cell lines were used for screening the hybrid supernatants : RAJI; LBF; WT47; TOK; SAVC (9th International Histocompatibility Workshop, held in Munich, West Germany, 1984). Table 2 contains the haplotypes of these cells as recorded in the literature.

Secondary testing was performed the day after the primary screen. A pool/mixture of cells (LBF, WT47, TOK, SAVC) was used in a negative selection process with all the supernatant being tested also against the immunizing cell. The purpose was two fold; to try and cut down the large number of hybrids that passed the primary screen while at the same time placing selection criteria based on specificity upon the hybrids. Quite simply, the haplotypes of the cell panel were not of interest and, thus, were selected against. Hybrids of interest included those that were producing antibodies against DR10, DR3 and selected DP epitopes. Hybrids that gave greater than 40% positive against RAJI and less than 10% positive against the cell mixture were selected for further testing. The percentage positive was calculated by the Bio-Rad Microplate Manager/PC, Data Analysis Software (Catalog 170-6617) (Bio-Rad, CA, USA).

Table 2 : HLA alleles of cell panel used in the secondary screen.

Cell Line	HLA Alleles					
	Class I			Class II		
	A	B	C	DR	DQ	DP β1/α1
RAJI*	23,30	8,13	--	3,10	2	α02
LBF	30	13	6	7	2	β1701 α0201
WT47	32	44	5	13	6	β1601 α01
TOK	24	52	--	15	6	β0901 α0201
SAVC	3	7	7	4	8	β1001 α0201

* Raji HLA type was obtained from Fisch, *et al.* (1989) and Soos, *et al.* (1993). All other cell line data came from the 11th International Histocompatibility Workshop, Volume 1 and the 9th International Histocompatibility Workshop and Immunobiology of HLA, Volume 1, Histocompatibility Testing 1987 and Histocompatibility Testing : Report on the Ninth International Histocompatibility Workshop and Conference, 1984.

-- indicates that particular typing is unavailable

3.11.8.C Tertiary Screening

The following homozygous cell lines were used for screening the hybrid supernatants that survived the secondary screen : RAJI; SAVC; COX; KAS116; YAR; SLE005; 8304 (11th International Histocompatibility Workshop number), (obtained from the 10th International Histocompatibility Workshop, held in New York City, USA, 1987). SAVC was again used to check the secondary screen. Table 3 contains the haplotypes of these cells as recorded in the literature. Raji's HLA type was obtained from Fisch, *et al.* (1989) and Soos, *et al.* (1993). All other cell line data came from the 11th International Histocompatibility Workshop, Volume 1 and the 9th International Histocompatibility Workshop, -- indicates that particular typing is unavailable

All hybridomas that had reaction patterns seen in Table 4 were selected for further screening. This selection process again eliminated all hybridomas that were of no interest, while at the same time allowing for a 'probable' specificity determination to be performed.

Table 3 : HLA alleles of cells used in the tertiary screen.

Cell Line	HLA Alleles					
	Class I			Class II		
	A	B	C	DR	DQ	DP β1/α1
RAJI*	23,30	8,13	--	3,10	2	β -- α02
SAVC	3	7	7	4	8	β1001 α0201
COX	1	8	7	3	2	β0301 α01
KAS116	24	51	--	1	5	β1301 α0201
YAR	26	38	--	4	8	β0401 α01
SLE005	2	60	10	13	6	β0301 α01
8304	--	--	--	--	--	β0401 α02

Note : "--" data unavailable

Table 4 : Hybridoma testing selection pattern for tertiary screen.

	CELL PANEL						POSSIBLE SPECIFICITY
	SLE	COX	KAS116	RAJI	8304	SAVC	
RX. PATTERN	+	+	---	+	+	---	DPβ1 69K
	---	---	+	+	+	---	DPβ1 55-57 (AAE/EAE) DPβ1 69K
	---	---	---	+	---	---	DR10
	---	---	---	+	+	---	DP

RX. : Reaction
Over 40% on a positive control report was considered (+).
Less than 10% on a positive control report was considered (---).
Possible specificity - refer to 11th International Histocompatibility Workshop, Volume 1, p.617. 8304 : 11th International Histocompatibility Workshop number.

3.11.8.D Quaternary Screening

The following cell lines were used for screening the hybrid supernatants : RAJI; WT51; DUCAF; BA1088; H0301; HAS301; PF04015; KAS011; VAVY; IBW9; TAB089; AMAI (obtained from the 9th International Histocompatibility Workshop, held in Munich, West Germany). All but two cell lines, BA1088 & RAJI, are homozygous. The BA1088 cell line originated from one individual whose B cells were transformed with the Epstein Barr Virus in this laboratory. This panel of cells allowed for a greater examination of specificity testing on the hybrids that survived the tertiary screen.

Table 5 shows the haplotypes of the cell panel used. All cell line data came from the 11th International Histocompatibility Workshop, Volume 1 and the 9th International Histocompatibility Workshop, -- indicates that particular typing is unavailable.

Table 5 : HLA alleles of cells used in the quaternary screen.

CELL LINE	HLA Alleles					
	CLASS I			CLASS II		
	A	B	C	DR	DQ	DP β1/α1
WT51	23	65	8	4	8	β02012 α01
DUCAF	30	18	5	17	2	β10202 α01
TAB089	2	46	11	8	6	β0202 α02022
HAS15	--	--	--	4	8	β0501 α --
VAVY	1	8	7	17	2	β0101 α0201
RAJI	23,30	8,13	--	3,10	2	-- α02
BA1088	29	7,17	--	3,10	--	--

Note "--" : data unavailable

3.12 Cell Freezing

At least 2 x 10^6 total cells/vial were frozen. Nunc vials were used (Nunc Tube, Gibco Laboratories, Grand Island, New York, USA). Cells were first counted as described before, and centrifuged at 1500 RPM for 10 minutes. 1 ml of freezing solution, 10% Dimethyl Sulfoxide (J.T.Baker Inc., NJ, USA) and 90% heat inactivated FBS (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada), was slowly added to resuspend the cell pellet. The freezing solution was kept at approximately 4^0C all the time. The cell suspension was then transferred to a chilled Nunc tube and immediately placed at -70^0C. These vials were later stored in liquid nitrogen.

3.13 Cell Thawing

Frozen cells vials were taken out of liquid nitrogen and placed on ice. These vial were then rapidly thawed by gently agitation in warm water (approx. 50^0C), containing common bleach, until there was only a crystal of ice remaining in the vial. The vial was them opened under aseptic conditions and the cell suspension pipetted into 10 mls of appropriate fresh medium. The cells were then washed twice at 1500 RPM for 10 min. and either placed in a 50 ml flask at a concentration of 3 x 10^5 cells/ml or a 24 well plate with 20% FBS (Gibco BRL, Canadian Life Technologies, Burlington, Ontario, Canada) if the viability was low.

3.12 Statistical Analysis

The following statistical testing methods were employed in the analysis of the *in vivo* results: two way factorial ANOVA, mixed between/within ANOVA, Dunnetts, Tukeys and post hoc analysis.

CHAPTER 4

RESULTS

4.1 The Effect of Administered Antibody on the Subsequent Serum Antibody Response

Based on published literature it was expected that IgM antibody administered before the injection of antigenic nucleated cells would increase the subsequent serum antibody response. Conversely, it was expected that IgG antibody should suppress the subsequent response. Before these experiments could be attempted, it was necessary to test the chosen antibodies, to select some suitable cells to use as antigen and to gather some baseline data on antigen dose response. In the next sections these preliminary studies are described.

4.1.1 Antibody Specificities and Purity Analysis

The antibodies have been described earlier with reference to class and specificity in the "Materials and Methods" section (Table 1, page 40). Before using them it was decided to check for any unanticipated cross reactions with mouse MHC

or other molecules. The antibodies were also checked by gel electrophoresis for purity.

4.1.1.1 Test for Cross-Reactivity of NFLD.M65C3 and NFLD.M68C11C8 with Mouse MHC and Human HLA-DR

Flow Cytometry was employed to test whether the monoclonal antibodies NFLD.M65C3, NFLD.M68C11C8 and DP139BD9 antibodies bind to mouse MHC Class II antigens as well as to the transfected human HLA-DP antigens. Each cell line was incubated with the appropriate antibody NFLD.M65C3, NFLD.M68C11C8 or DP139BD9 then with specific conjugate, phycoerythrin labelled goat anti-mouse IgG or IgM. Analysis was done on a FACStar^{plus} Flow Cytometer.

Figure 5 show the results of experiments performed to investigate whether binding of the NFLD.M65C3 and NFLD.M68C11C8 is directed against the transfected Human HLA-DP antigens or if there is cross-reactivity with mouse MHC and Human HLA-DR. Graph A shows cell lines tested against NFLD.M68C11C8. Cell line L556 is a mouse fibroblast transfected with Human HLA-DR, TIB209 is a mouse cell line expressing IgM and mouse MHC only and L25.4 represents a mouse cell line transfected with Human HLA-DP. As compared to the control there is no binding to L556 or TIB209. There is significant binding to L25.4. Graph B shows cell lines tested against NFLD.M65C3. Cell line A20 represents a mouse B cell expressing IgG

and mouse MHC only. As compared to the control there is no significant binding to A20 or L556. There is, however, significant binding to L25.4. Graph C shows cell lines tested against DP139BD9 (non-specific mouse IgM). As compared to the control neither cell line binds particularly well with DP139BD9. Thus, these results indicate that NFLD.M65C3 and NFLD.M68C11C8 are binding only to the human HLA-DP MHC molecules that are being expressed on the murine fibroblasts.

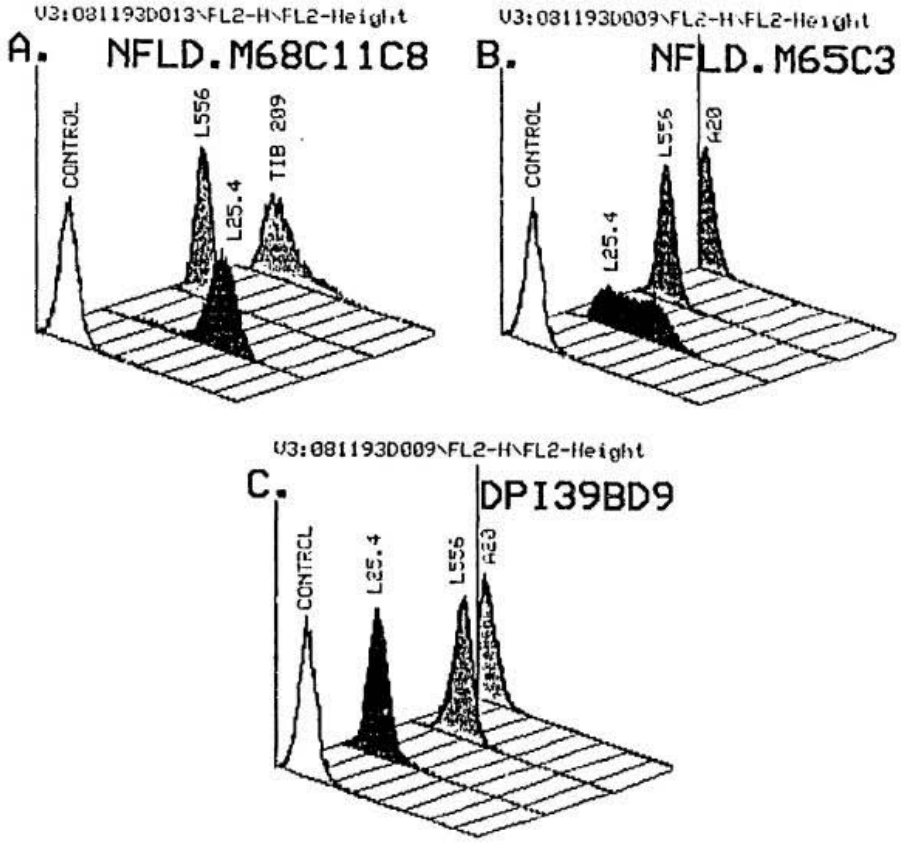

Figure 5 : FACS analysis to test for cross-reactivity of NFLD.M65C3 (IgM), NFLD.M68C11C8 (IgG) and DPI39BD9 (non-specific IgM) antibodies with mouse MHC. L556 is a mouse fibroblast transfected with human HLA-DR; L25.4 is a mouse cell line transfected with human HLA-DP; TIB209 is a mouse B cell line expressing IgM and mouse MHC; A20 a mouse B cell expressing IgG and mouse MHC only. Controls -- mouse L cell (expressing Class I MHC). This represents a three dimensional plot of red florescence in log scale.

4.1.1.2 Test for Antibody Homogeneity

The next task was to grow the antibody secreting hybridomas, concentrate the supernatant, purify and check for homogeneity. All antibodies were purified by agarose gel affinity columns. In the case of the NFLD.M65C3 and DP139BD9, which are IgM antibodies, the concentrated overgrown supernatants were passed through anti-mouse IgM (μ-chain specific) - agarose columns for purification. The NFLD.M68C11C8, an IgG antibody, was purified by the Affi-Gel Protein A MAPS II Kit. After the correct amount of antibody was purified, gel electrophoresis (SDS-PAGE) was performed to check for purity/homogeneity. Figure 6 represents SDS-PAGE performed on samples of NFLD.M65C3 and NFLD.M68C11C8. The molecular weight markers shown in Figure 6 consisted of markers from 14 400 MW to 97 400 MW. All the antibodies were first checked in this manner before *in vivo* experiments were performed. In this figure, one can see the heavy and light chains of NFLD.M68C11C8 (Lane 1) and NFLD.M65C3 (Lane 2). Upon comparison to the molecular weight marker (Lane 3) the NFLD.M65C3 lane shows a clean sample with distinct bands indicating the heavy chain between 97.4 Kd and 66.2 Kd and the light chain at approximately 29 Kd. The NFLD.M68CLLC8 lane displays a distinct light chain band at approximately 27 Kd but the presence of a broad band or bands in the heavy chain region at approximately 51Kd. This may indicate the possibility that the sample is a biclonal or oligoclonal as opposed to a monoclonal IgG antibody.

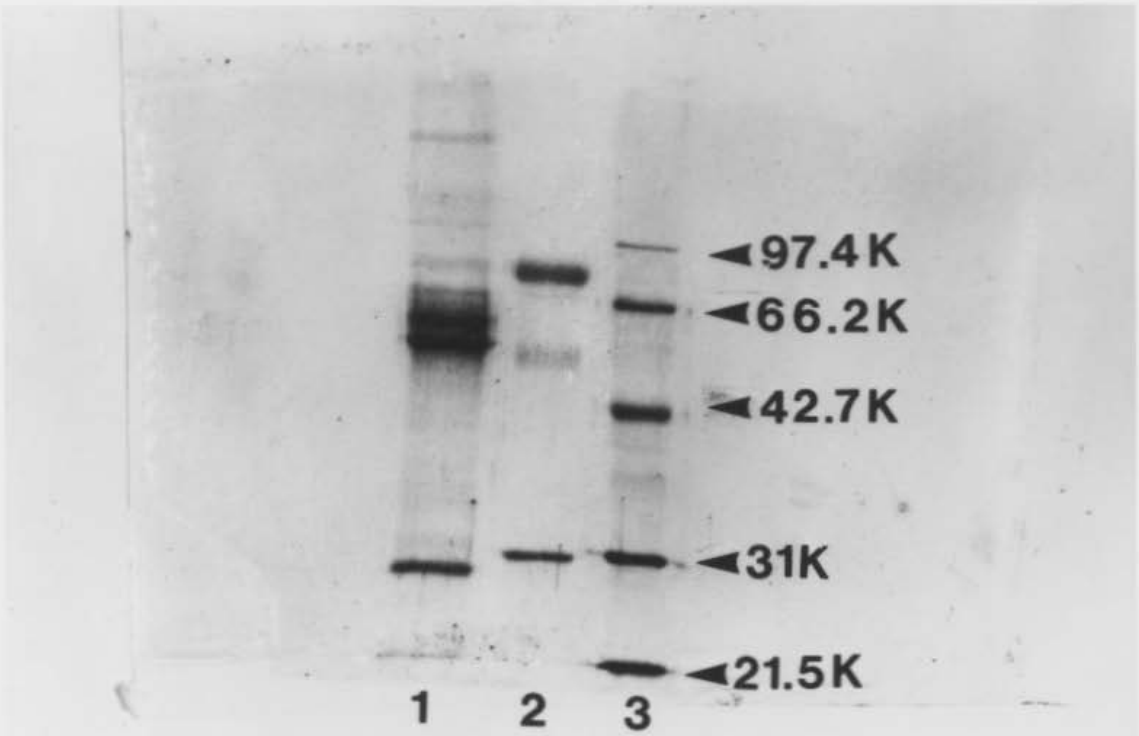

Figure 6 : SDS-PAGE analysis of NFLD.M65C3 and NFLD.M68C11C8 antibodies. Lane 1 : NFLD.M68C11C8, Lane 2 : NFLD.M65C3 and Lane 3 : Molecular weight markers. Bands were visualized via Coomassie brilliant blue.

4.1.2 Selection of Antigens

The next task was to select antigens to be used in the *in vivo* experiments to follow. It was decided that a human B cell line and a mouse fibroblast transfected with human HLA-DP should be selected. The human B cell line, Raji, was chosen based on work prior to this project (Samuels, 1992). This Honours Thesis had considered such things as cell culture viability, expression of Class II antigens and binding of varied cell lines to the pan DP antibodies of IgM (NFLD.M65C3) and IgG (NFLD.M68C11C8) classes. The transfected mouse fibroblast provided human HLA-DP molecules which would be recognized as foreign against a background of self cells, thus, the enhancement/suppression response would be directed specifically to HLA-DP determinants. However first, the most suitable transfectant had to be chosen.

A panel of HLA-DP transfected mouse fibroblasts (Table 6) were tested against purified IgM (NFLD.M65C3) and IgG (NFLD.M68C11C8) by ELISA. Prior to these and subsequent experiments a standardization of antibodies waas performed. The minimum dilution that resulted in a significant absorbance at 490nm was selected. The transfectants were used 24 hours after removal from the plastic culture dishes by trypsin treatment. The ELISA was replicated, the pattern remaining consistent (Table 6). NFLD.M67 (IgG) has been extensively tested on a number of cell lines and mouse transfected mouse fibroblasts in this laboratory and has been found to react with a monomorphic determinant of HLA-DP, thus, this antibody was used as a positive

control. The selection criterion was basic : to identify a cell line that bound well with the antibodies of interest, specific IgM (NFLD.M65C3) and specific IgG (NFLD.M68C11C8). Based on these tests it was shown that the 8305 (L25.4) transfectant had the strongest binding with both IgM (NFLD.M65C3) & IgG (NFLD.M68C11C8). Thus, this cell line was chosen for the *in vivo* experiments to follow.

Table 6 : ELISA results from a panel of six HLA-DP transfectants tested against NFLD.M65C3 and NFLD.M68C11C8 antibodies.

CELL LINE	ANTIBODY TESTED (O.D. 490 nm)			
	10% FBS DMEM	NFLD.M67	NFLD.M65C3	NFLD. M68C11C8
8305*	0.175	2.214	1.486	1.858
DPβ1301α01	0.157	2.112	0.988	1.398
8304*	0.178	1.940	0.611	0.901
DPβ0301α01	0.192	2.154	0.836	1.405
DPβ0202α01	0.184	1.871	0.702	0.937
DPβ0501α02	0.164	1.535	0.502	0.723

* 11th International Histocompatibility Workshop numbers. Parental cells for all these lines were mouse L cells. All cells are mouse fibroblasts that were transfected with Human MHC, HLA-DP. Refer to Table 1 for specificity of NFLD.M65C3, NFLD.M67 and NFLD.M68C11C8 antibodies. NFLD.M67 : Positive Control 10% FBS DMEM : Negative Control (Blank)

4.1.3 Selection of Non-Specific IgM Antibody

It was not until the antigens had been selected that the non-specific IgM antibody could be chosen.

Thus, the purpose of this experiment was to choose an IgM monoclonal immunoglobulin that was non-reactive with the antigen of choice 8305 (L25.4). A panel of IgM monoclonal antibodies were tested against two mouse fibroblasts transfected with Human HLA-DP, the 8306 cell line was known to react poorly with HLA-DP specific IgM (NFLD.M65C3) in past experiments so was included as a "control". These results were replicated over a number of assays, each consistently giving the same pattern of binding. From the results, Table 7, it can be seen that one of these (DP139BD9) gave the weakest binding to 8305. As a result, it was chosen to be used in the *in vivo* experiment as the non-specific IgM antibody.

Table 7 : ELISA results from a panel of IgM antibodies tested against 8305 and 8306 transfectant cell lines.

ANTIBODY TESTED	CELL LINE (O.D 490nm)	
	8305*	8306*
10% FBS DMEM	0.352	0.411
NFLD.M67	2.110	2.481
NFLD.M65C3	1.987	0.752
DP168EH1	0.221	0.249
DP169GE7	0.255	0.289
DP143GG4C3B5	0.532	2.588
NFLD.M53F69DH3H8	0.219	0.279
DP139BD9	0.210	0.319

GAM - IgM specific conjugate was used in the assay. Mouse fibroblast were transfected with Human MHC, HLA-DP. * 11th International Histocompatibility Workshop numbers. NFLD.M67 : Positive Control. HLA-DP specific IgM (NFLD.M65C3) was included again to recheck/check the binding with 8305, therefore, also acting as a positive control. 10% FBS DMEM was used as the Negative Control (Blank).

4.1.4 Antigen Dose Response

Experiments were conducted to determine the 'optimal' dose of antigen for the *in vivo* experiments to follow. The 'optimal' dose in these experiments would fulfil two requirements; (1) the dosage would be one that did not result in a maximum response such that the IgM enhancement could not be observed, and (2) conversely, a dose in which the IgG suppression could be observed. These experiments were quite straightforward. Groups of mice were injected with varying amounts of cells and serum was collected at various time points. CELISA assays were then performed on these samples so as to quantify the levels of antibody specific to the injected antigen.

A. RAJI

The purpose of these experiments was to determine the 'optimal' dose of Raji for the *in vivo* studies to follow. The experiment consisted of groups of 4 C3H mice injected intravenously with varying amounts of Raji cells; Group A : 5.0×10^5, Group B : 1.0×10^6, Group C : 5.0×10^6, Group D 1.0×10^7 and Group E : 2.0×10^7. A control group of mice was injected with sterile PBS. Blood and then serum samples were collected from the animals on day 0, 8 and 14 and a CELISA was performed, as seen in Figure 7. Time 0 represents the initial/existing levels of antibody in the animals 2 hours prior to experimentation. It can be seen that with increasing doses of injected cells, there was an increase in the subsequent antibody response each time; no

plateau was reached in this dose range. A dose of 5 x 10^6 Raji cells/mouse was determined to be used, as it allowed room for both an increase and a decrease to be detected resulting from antibody treatments. Standard error bars represent values obtained from the four mice comprising each group.

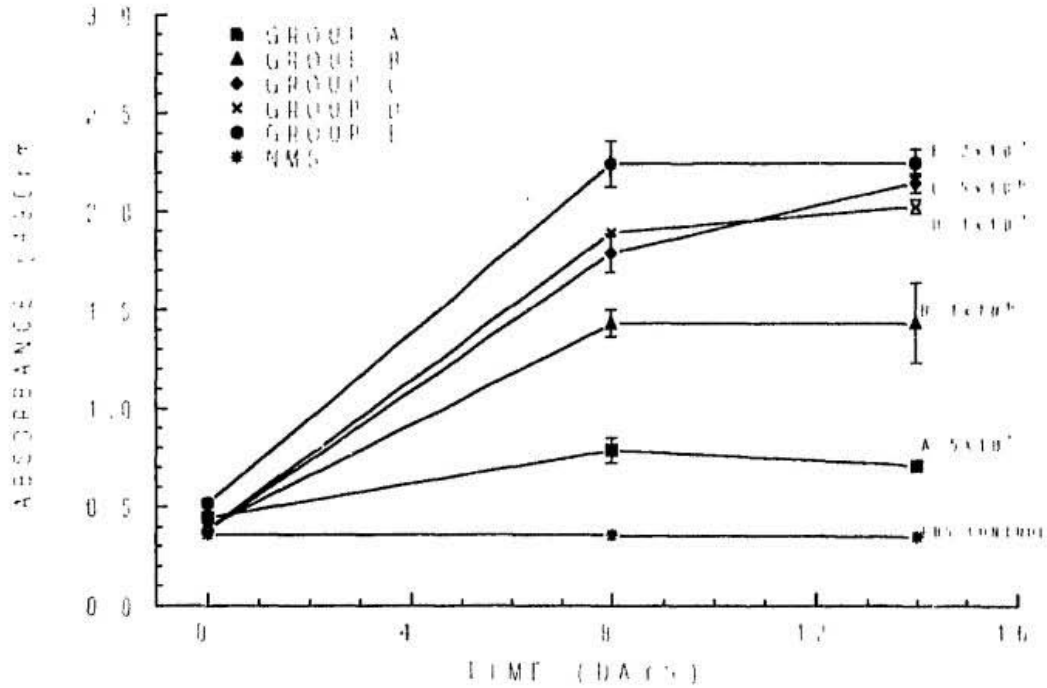

Figure 7 : CELISA results of groups of C3H mice injected with differing doses of Raji cells. Note : each treatment group consisted of 4 mice; serum samples were collected at day 0, 8 and 14; graph represents antibody (1/800 dilution) specific for the immunizing cell. Doses consisted of : Group A - 5.0 x 10^5 cells/animal, Group B - 1.0 x 10^6 cells/animal, Group C - 5.0 x 10^6 cells/animal, Group D - 1.0 x 10^7 cells/animal, Group E - 2.0 x 10^7 cells/animal, a control group - sterile PBS. Standard error bars represent values obtained from different mice.

B. L25.4

A similar experiment was also performed for the L25.4 cell line. Consideration of the selection of the 'optimal' dose of L25.4 cells was identical as in the case of the Raji cell line (4.1.4 Part A).

The experiment consisted of four groups of 4 C3H mice injected intravenously with varying numbers of L25.4 cells : Group A received 5.0 x 10^6, Group B received 1.0 x 10^7, Group C received 2.5 x 10^7, and an additional group of mice were injected with sterile PBS. Blood/serum samples were collected from the animals on day 0, 6 and 12 (based on previous work (Samuels, 1992)) and a CELISA performed as seen in Figure 8. Time 0 represents the initial/existing levels of antibody in the animals 2 hours prior to experimentation. At Day 6 there is an inverse dose response (based on the days assayed), animals receiving the smallest dosage 5.0 x 10^6 cells per animal displayed the strongest response followed by the 1.0 x 10^7 cells/animal and 2.5 x 10^7 cells/animal treated groups, respectively. Higher doses of 2.5 x 10^7 cells/mouse were attempted, but the survival of the animals was jeopardized (i.e the animals died). The graphs show an unexpected reduction in the serum antibody response with increasing doses of these transfectant cells. A dose of 5.0 x 10^6 L25.4 cells/mouse was selected for the *in vivo* experiment.

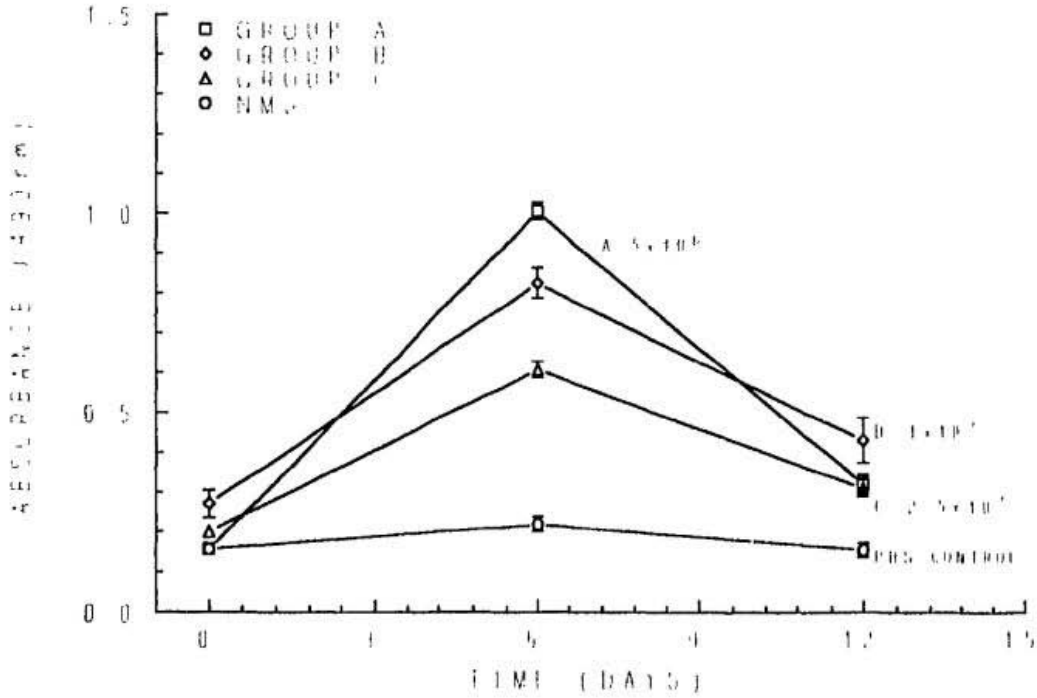

Figure 8 : CELISA results of groups of C3H mice injected with differing doses of L25.4 Cells. Note : Each group consisted of 4 mice; serum samples were collected at day 0, 6 and 12, graph represents antibody specific for the immunizing cell (1/200 dilution). Doses consisted of : Group A - 5.0 x 10^6 cells/animal, Group B - 1.0 x 10^7 cells/animal, Group C - 2.5 x 10^7 cells/animal, a control group - sterile PBS. Standard error bars represent values obtained from different mice.

4.1.5 *In Vivo* Experiments

With all the experimental conditions set, the next step was to determine if IgM enhancement and IgG suppression could be observed with the nucleated antigens that had been selected. In all experiments the antibody was administered intravenously 1-2 hours prior to intravenous injection of antigen. All treatment groups consisted of 4 C3H mice. All mice were bled, sera obtained and a CELISA performed on the serum samples in order to quantify the primary antibody response directed against the immunogen. NMS (normal mouse serum) represents sera obtained from all mice prior to any manipulation.

4.1.5.1 Pretreatment by HLA-DP specifc IgM (NFLD.M65C3) and HLA-DP specific IgG (NFLD.M68C11C8) with Raji as Antigen

In the first experiment the antigen used was the human B cell line, Raji. The experiment consisted of 4 groups of mice in which the antibody dose was determined based on experiments contained in the literature (Henry and Jerne, 1968). Group A received HLA-DP specific IgM (NFLD.M65C3) (0.42mg/mouse) I/V, Group B received HLA-DP specific IgG (NFLD.M68C11C8) (0.41mg/mouse) I/V, Group C received sterile PBS and another group, AB Alone, group received only HLA-DP specific IgM (NFLD.M65C3) antibody. The NMS group represents the average (O.D) of sera taken from all mice 24 hours prior to any manipulation. After 1.5 hours these

mice were injected with 5 x 10^6 Raji cells, I/V. At day 7, serum was collected from each mouse and a CELISA performed. For each group IgM and IgG levels were individually detected by the use of a class specific conjugate, represented on the graphic as M and G respectively. The AB Alone group represents the presence of HLA-DP specific IgM (NFLD.M65C3) antibody at day 7 in mice not receiving antigen, thus indicating the rate at which specific IgM antibody disappears from the circulation. It should be noted that the original design of this experiment included a group of mice receiving only HLA-DP specific IgG (NFLD.M68C11C8) antibody. However, this particular batch of antibody was found to be bacterially contaminated after the experiment was conducted, thus, these data have been omitted.

Mice treated with HLA-DP specific IgM (NFLD.M65C3) did show an enhanced primary response both in the IgM and IgG antibody levels at day 7, as compared to the PBS control group (as illustrated in Figure 9). Statistically, this difference was significant ($p<0.001$, two way factorial ANOVA). The IgG treated group did show a lowered response in both IgM and IgG levels as compared to the PBS control group, however, this difference was not statistically significant ($p>0.05$, two way factorial ANOVA). The response displayed by the IgM pretreated animals is in line with the results published to date in the literature (see introduction).

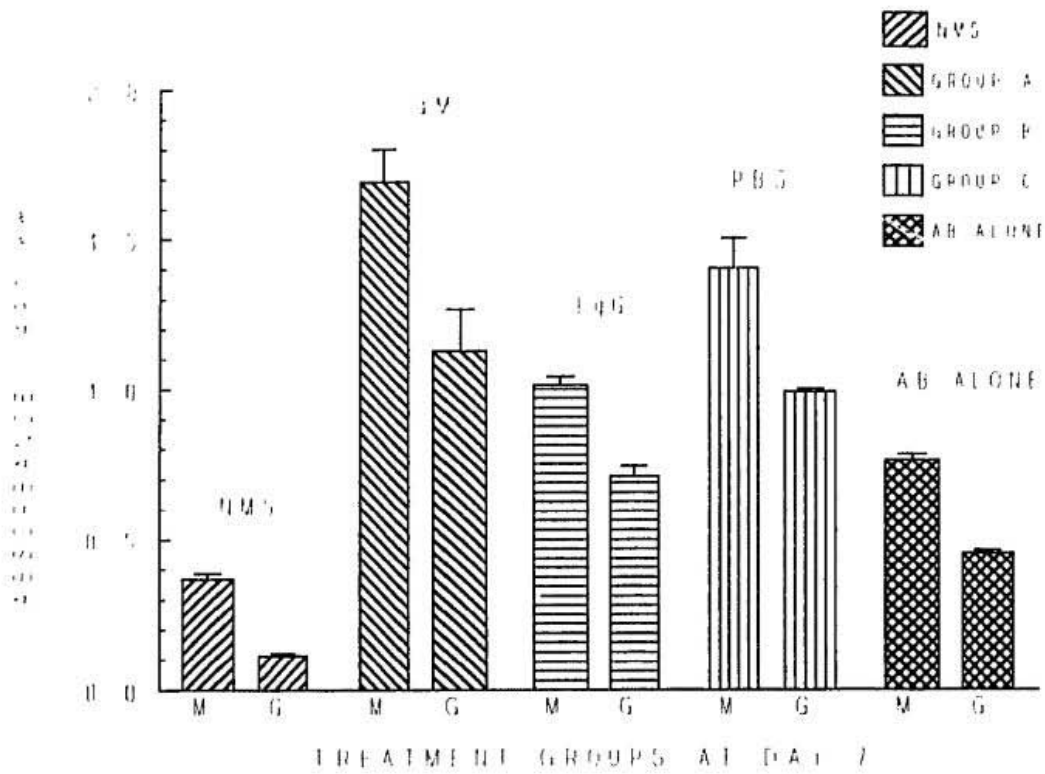

Figure 9 : CELISA results of sera obtained at day 7 of C3H mice pretreated with HLA-DP specific IgM (NFLD.M65C3), HLA-DP specific IgG (NFLD.M68C11C8) and sterile PBS prior to administration of 5 x 10^6 Raji cells/animal. AB ALONE group represents animals only receiving HLA-DP specific IgM (NFLD.M65C3) antibody. NMS represents an average of sera obtained from every animal 24 hours prior to any manipulation. Treatments : Group A - NFLD.M65C3, Group B - NFLD.M68C11C8, Group C - sterile PBS. M and G represent, respectively, IgM and IgG specific conjugate.

4.1.5.2 Pretreatment by HLA-DP Specific IgM and HLA-DP Specific IgG with HLA-DP Transfectant as Antigen

In this experiment the enhancement/suppression phenomena were tested in a similar experiment (Figure 9) with the DP transfectant (L25.4) cells as the antigen. Group A received HLA-DP specific IgM (NFLD.M65C3, 0.34mg/mouse) I/V, Group B received HLA-DP specific IgG (NFLD.M68C11C8, 0.30mg/mouse) I/V, and Group C received sterile PBS. Prior standardization experiments resulted in a alteration of antibody dosage in comparison to the previous experiments. This difference may be attributed to the differing antigens. After 2 hours these mice were injected with 5 x 10^6 transfectant cells, I/V. On days 6, 9, 12 and 16 sera were collected from each mouse and a CELISA performed. The AB Alone group represents the presence of residual HLA-DP specific IgM antibody (NFLD.M65C3) in circulation of mice receiving antibody only. NMS represents an average of sera obtained from every animal 24 hours prior to any manipulation. It should again be noted that the original design of this experiment included a group of mice receiving HLA-DP specific IgG (NFLD.M68C11C8) antibody only. However, this particular lot of antibody was found to be bacterially contaminated after the experiment was conducted, thus, this data has been omitted.

The IgM pretreated group showed an enhanced response from day 6 to 16 compared with the PBS treatment group (Figure 10). The IgG pretreated group's

response to the immunogen was less than that of the PBS control at all times assayed. The antibody alone group allowed for detection of the antibody decay within the mice. The NMS group allowed for a baseline prior to experimentation and this value was determined by averaging data from all the mice sera prior to experimentation. In terms of O.D units there was an increase of about fourfold due to the pretreatment with specific IgM antibody and a decrease of approximately 50% due to the pretreatment with specific IgG antibody on day 6. The maximum enhancement by IgM was observed on Day 6. The maximum suppression by IgG was observed on Day 9.

Treatment and days, as well as the interaction between the two, were all significant ($p<0.001$, mixed between/within ANOVA). Since the ANOVA analysis indicated a significant interaction, a detailed post hoc analysis was performed. It was found that the HLA-DP specific IgM (NFLD.M65C3) and HLA-DP specific IgG (NFLD.M68C11C8) treated groups were significantly different from the PBS control on all three days tested ($p<0.01$, Tukeys).

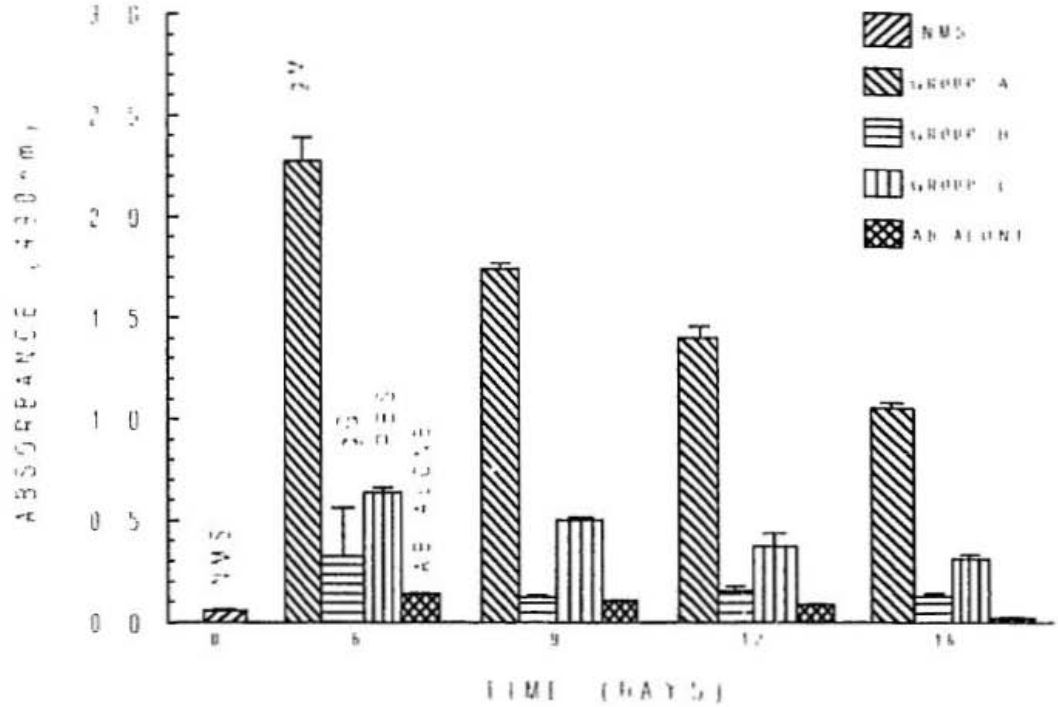

Figure 10 : CELISA results of C3H mice pretreated with HLA-DP specific IgM (NFLD.M65C3), HLA-DP specific IgG (NFLD.M68C11C8) and PBS prior to administration of 5 x 10^6 cells/animal DP transfected fibroblasts (L25.4). Serum samples were taken at Day 0, 6, 9, 12 and 16. Treatments : Group A - specific IgM, Group B - specific IgG, Group C - Sterile PBS. NMS represent normal mouse serum and the AB Alone group represents the antibody decay (animals receiving only HLA-DP specific IgM antibody, NFLD.M65C3). Mixed IgM and IgG specific conjugate was used.

4.1.5.3 Pretreatment by HLA-DP specific IgM and Non-specific IgM with HLA-DP transfected fibroblast as Antigen

As presented in the introduction, the literature suggests that one of the characteristics of IgM enhancement is that the response is antigen (particle) specific but not epitope specific. In order to verify that this, an experiment was performed in which animals were pretreated with non-specific IgM, DP139BD9, and HLA-DP specific IgM antibody (NFLD.M65C3) before immunization. This information was important for the planning of experiments described in the second section of this thesis.

From Henry's and Jerne's (1968) experiment the results clearly indicated that as the animals received increasing amounts of IgM antibody, the primary response became stronger and stronger. It was decided to repeat the previous experiment adding an extra group, that being non-specific IgM (Figure 10), and, in an attempt to improve on the tremendous enhancement seen in the previous study, the animals were administered approximately twice as much antibody. Group A received HLA-DP specific IgM (NFLD.M65C3, 0.68mg/mouse) I/V, Group B received a non-specific IgM (DP139BD9, 0.62mg/mouse) I/V, and Group C received sterile PBS. After 1.5 hours these mice were injected with 5×10^6 L25.4 cells, I/V. On days 6, 10 and 15, serum samples were collected from each mouse and a CELISA performed. AB Alone group represents the presence of antibody in mice only receiving HLA-DP specific IgM (NFLD.M65C3) antibody.

The NFLD.M65C3 pretreated group did experience an increase in the response to L25.4 as compared to the PBS control group (Figure 11). Unexpectedly, the DP139BD9 pretreated group also experienced an increase in the response to L25.4, as compared to the PBS control. However, this response was not as strong as the HLA-DP specific IgM (NFLD.M65C3) administered group. The NMS group is the baseline obtained from all animals prior to experimentation. The Antibody Alone group represents the decay of HLA-DP specific IgM (NFLD.M65C3) antibody within the mice.

Statistically, both treatment and days had a significant effect on outcome ($p<0.001$, mixed between/within ANOVA). However, there was no significant interaction ($p>0.05$, mixed between/within ANOVA). Post hoc analyses were therefore performed separately for treatment and days. For treatment, both the specific IgM and non-specific IgM differed from the PBS control ($p>0.01$, Dunnets). For days, day 6 differed from day 10 ($p<0,05$, Tukeys); day 6 differed from day 15, and day 10 differed from day 15 ($p<0.01$, Tukeys).

This study differed from the previous experiment in two significant ways : a) twice as much specific IgM antibody was used, b) a non-specific IgM antibody treated group was included. Surprisingly, doubling the specific IgM antibody concentration did not increase the enhancement seen in the previous experiment. Rather, upon comparison the relative enhancement diminished. This lower response may be due to

the existence of an optimum of administered IgM antibody and a concentration above this does not aid in enhancing the primary response. In other words, the dose of the IgM antibodies may have been too high. When comparing the other groups of the two experiments both the PBS control groups gave similar optical density readings (approx. 0.6 O.D), however, the AB Alone group, representing the antibody decay, produced an optical density approximately 4.7 times higher than the previous experiment (0.7 vs. 0.15 O.D). Since twice as much antibody was used, this group was expected to give higher an absorbance reading perhaps twice as high, than seen in the prior experiment but not 4-7 times as high. Standard error bars represent values obtained from different mice.

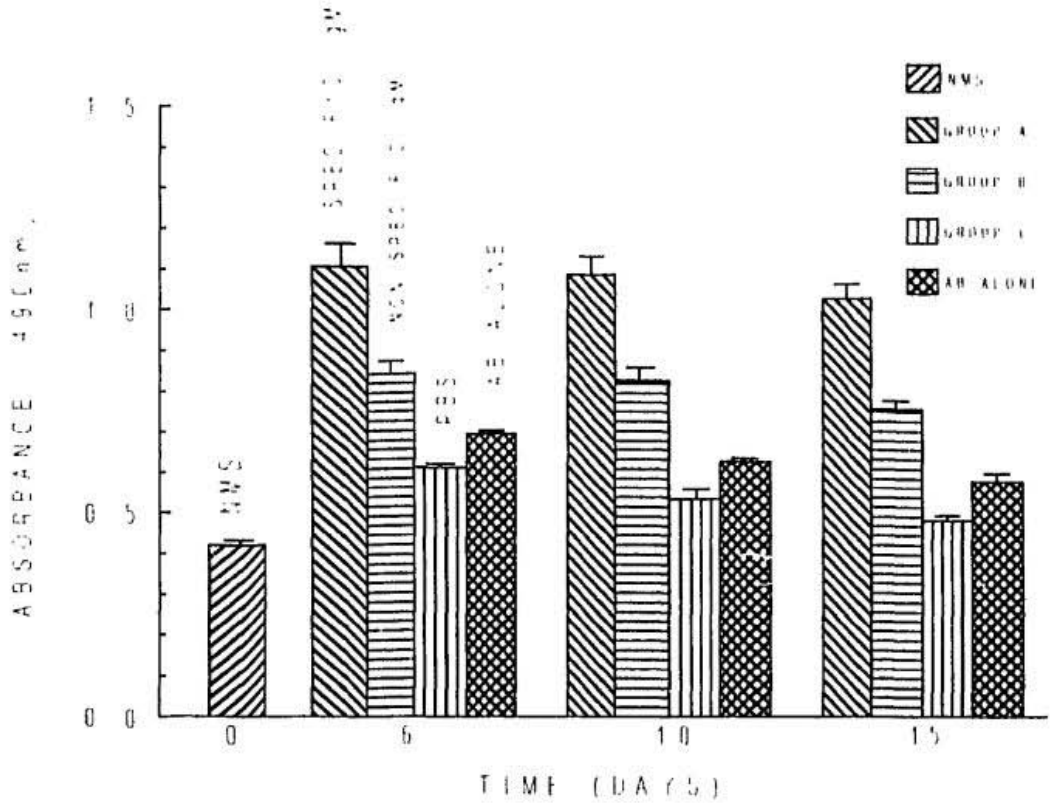

Figure 11 : CELISA results of C3H mice pretreated with HLA-DP specific IgM, non-specific IgM and PBS prior to administration of 5 x 10^6 L25.4 cells/animal. Serum samples were taken at Day 0, 6, 10 and 15. Treatments : Group A - HLA-DP specific IgM (NFLD.M65C3), Group B - non-specific IgM (DP139BD9), Group C - sterile PBS. NMS represent normal mouse serum prior to manipulation and the AB Alone group represents the antibody decay (animals receiving only DP specific IgM - NFLD.M65C3).

4.2 Effect of Administered Antibody on the Population of Hybridomas Harvested 3 Days After Antigen.

The following experiments were conducted base on the hypothesis that the IgM antibody could influence/enhance the production of hybridomas with specificities that our laboratory was interested in developing. These "hybridomas of interest" are specific for HLA-DR and HLA-DP molecules. This laboratory is particularly interested in obtaining hybridomas against; DPβ1 69(K), DPβ1 55-57(AAE/EAE) and DR10. Thus, the idea was to use as an immunogen, a cell line containing the aforementioned sequences. Before these experiments could be attempted, it was necessary to determine the full HLA type of the chosen immunogen, Raji cells, and to determine a dosage for the subsequent experiments to follow. These experiments, in conjunction with the results obtained from the previous *in vivo* studies, provided the information necessary to design the fusion experiments. In the next section the preliminary studies are described.

4.2.1 HLA Type Determination of Raji

HLA typing of Raji cells was carried out by (1) Complement Dependent Cytotoxicity Typing Plates and (2) HLA-DP typing via a monoclonal antibody panel. The partial HLA type of Raji cells has been published. These experiments were conducted in order to confirm the reports and to fill in the 'gaps' in these reports. It was also necessary to know the HLA type of the immunogen so as to choose cell lines with

appropriate HLA types for the screening process that would determine the various specificities of the resultant hybridomas.

4.2.1.1 HLA Type Determination via CDC Typing Plates

From previous work by Fisch, *et al.* (1989) and Soos, *et al.* (1993) it was stated that the HLA type of Raji cells was A 23,30; B 8,13; C not typed; DR 3,10 52; DQ 2; DP unknown.

Complement dependent cytotoxic (CDC) HLA typing plates obtained from the Canadian Red Cross Society were used to verify the claims presented in the literature and to identify the C locus.

The reading/scoring of these plates/wells was particularly difficult. The typing was attempted three times each with varying results. As a result the A and B locus could not be typed with certainty due to irregularity. The problems were a combination of inexperience, dead cells and complement toxicity. More experienced people in the laboratory also typed Raji cells but met with the same lack of success. However, the DR 10 and DRw52 types did remain consistent. Also, with respect to the C locus, the Cw3 haplotype was consistent in all the experiments.

4.2.1.2 HLA-DP Determination via Monoclonal Antibodies

DP typing was not available with the Canadian Red Cross Society's CDC typing plates. For investigating the HLA-DP type, monoclonal antibodies produced in this laboratory were used. These antibodies recognize known epitopes of expressed DP α and β chains. Table 8 shows the DPβ1 amino acid sequences associated with their respective HLA-DP alleles and the monoclonal antibodies used, with their respective specificities. For example, the M120 antibody recognizes an epitope conferred by the "GPM" motif at amino acid 85-87 and expressed by the DPβ1 1501, 1801, 2801, 3401 alleles. Thus, this antibody will bind to cells expressing any of these "DP types".

A CELISA, testing these monoclonal antibodies against the Raji cell line was then performed as seen in Table 9. Additional monoclonal antibodies were tested. along with some of the monoclonals previously tested in the CELISA, using FACS analysis (as seen in Figure 12). For both assays the positive and negative controls were identical; NFLD.M67 (an antibody against monomorphic HLA-DP determinants) was used as the positive control, while 10% FBS DMEM served as the negative control. An absorbance reading (490nm) greater or equal to 1.0 was considered as positive. As can be seen from the readings, the difference between positive absorbance readings (eg. NFLD.M118 : 2.24) and negative readings (eg. NFLD.M73 : 0.106) was usually large (Table 9). Table 10 summarizes the results of all the monoclonals tested against Raji. NFLD.M66, NFLD.106, NFLD.M117 and NFLD.M118 antibodies were tested by CELISA and FACS with both assays producing the same result. Additional antibodies tested by FACS included NFLD.M111, NFLD.M114, NFLD.M118, GGPM (Table 10). The associated

epitopes for these antibodies as well as possible DP types are listed in Table 8. For example, the M58 epitope is DE at position 55-56 β1 chain. In reference to published sequences, this epitope is present in DPβ1 0201, 0301.....3501. Therefore, this antibody will bind to cells expressing any of these DP types. Similarly for the rest of the antibodies in Table 8.

The monoclonal antibodies with mapped epitopes could then be matched with known HLA-DP types (Table 8). By comparing the results of the monoclonal antibody panel from both the CELISA and FACS assays, one could eliminate a number of HLA-DP and epitope possibilities. From Table 9 and Figure 12, Raji was positive (bound to) monoclonals NFLD.M66 (DEAV, 84-87), NFLD.M70 (DPα02) and NFLD.M118 (DPα02) but negative against the remaining panel. Based on comparisons within Table 8, Raji can be partially typed, with respect to HLA-DP, as having one (homozygous) or any two in combination (heterozygous) of the following HLA types α : 2; β : 0101, 1101, 1301, 2101, 2701, 3001.

With respect to the monoclonal antibodies that reacted positively with Raji cells the related epitopes can also be examined (Table 11). Table 11 presents the possible HLA-DPβ1 types with associated epitopes after the positive and negative reacting monoclonal antibodies to Raji cells have been cross checked (within Table 8). By this method Raji cells could be partially epitope typed as having, at positions 84-87 DEAV. With respect to the other hypervariable regions, of interest, the possibilities were

narrowed down to :

35-36 : YA

55-57 : AAE or EAE

69 : K, R or E.

84-87 : DEAV (definite).

Table 8 : DPβ1 amino acid sequence variations and their associated haplotypes.

AMINO ACID SEQUENCE HLA-DPβ1						
DE	DEE	DED	DEAV	GGPM	GPM	LV/FV
55-56	55-57	55-57	84-87	84-87	85-87	35-36
MONOCLONAL ANTIBODIES						
M58	M106	M111	M66	MYE901	M120	M117
M60		M115		GGPM		M77
M73		M119				
M101						
M112						
POSSIBLE HLA-DP TYPE						
0201	0201	0301	0101	0201	1501	0201
0301	0402	0601	0301	0202	1801	0202
0402	0801	0901	0501	0401	2801	0401
0601	1001	1401	0601	0402	3401	0402
0801	1601	1701	0801	3201		0501
0901	1801	2001	0901	3301		0801
1001	2801	2901	1001			1601
1401		3501	1101			1901
1601			1301			2201
1701			1401			2301
1801			1601			2401
2001			1701			2801
2501			1801			3101
2801			1901			3201
2901			2001			3301
3201			2101			3401
3501			2701			
			2901			
			3001			
			3101			
			3501			

M* antibodies refer to NFLD.M## monoclonal antibodies.
α 01 antibodies : NFLD.M114 & NFLD.M116
α 02 antibodies used : NFLD.M118 & NFLD.M70
From 11th International Histocompatibility Workshop and Conference, Volume 1, p.615-618.

Table 9 : CELISA results of a panel of monoclonal antibodies against the RAJI cell line.

ANTIBODY TESTED	ABSORBANCE (490nm) RAJI CELL LINE
NFLD.M67	1.480
10%FBS DMEM	0.110
NFLD.M58	0.095
NFLD.M60	0.091
NFLD.M66	0.460
NFLD.M70	0.437
NFLD.M73	0.147
NFLD.M77	0.117
NFLD.M106	0.106
NFLD.M112	0.097
NFLD.M115	0.105
NFLD.M116	0.106
NFLD.M117	0.121
NFLD.M118	2.240
NFLD.M119	0.129
NFLD.M120	0.110
MYE901	0.0977

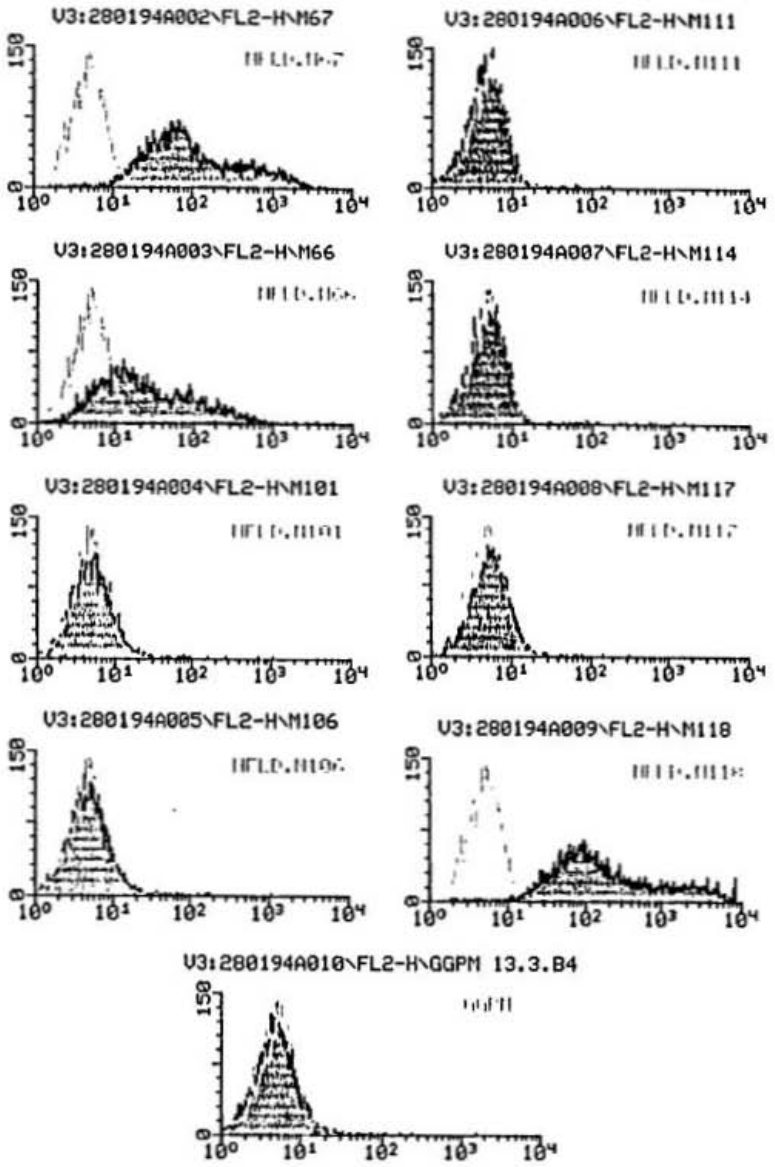

Figure 12 : Flow Cytometry results of various monoclonal antibodies tested against the Raji cell line. NFLD.M67, an antibody against monomorphic HLA-DP determinants, was used as the positive control, while 10% FBS DMEM served as the negative control.

Table 10 : Summary of Flow Cytometry and CELISA results in the investigation of Raji's HLA-DP type by monoclonal antibodies.

ANTIBODY TESTED	RESULTS (POSITIVE/NEGATIVE)	
	CELISA	Flow Cytometry
NFLD.M58	NEG	NT
NFLD.M60	NEG	NT
NFLD.M66	NEG	NEG
NFLD.M70	POS	NT
NFLD.M73	NEG	NT
NFLD.M77	NEG	NT
NFLD.M106	NEG	NEG
NFLD.M112	NEG	NT
NFLD.M115	NEG	NT
NFLD.M116	NEG	NT
NFLD.M117	NEG	NEG
NFLD.M118	POS	POS
NFLD.M119	NEG	NT
NFLD.M120	NEG	NT
MYE901	NEG	NT

Table 10 : cont'd

ANTIBODY TESTED	RESULTS (POSITIVE/NEGATIVE)	
	CELISA	Flow Cytometry
NFLD.M111	NT	NEG
NFLD.M114	NT	NEG
GGPM	NT	NEG

NOTES :

NEG : Negative

POS : Positive

NT : Not Tested

The results have been compiled from Table 9 and Figure 12.

Table 11 : Summary of possible HLA-DPβ1 epitopes of Raji cell line as a result of monoclonal antibody testing by Flow Cytometry and CELISA.

POSSIBLE HLA-DPβ1 TYPE	POSSIBLE HLA-DPβ1 ASSOCIATED EPITOPES (VARIATION OF AMINO ACIDS)			
	35-36	55-57	69	84-87
0101	YA	AAE	K	DEAV
1101	YA	AAE	R	DEAV
1301	YA	AAE	E	DEAV
2101	LV	EAE	E	DEAV
2701	YA	AAE	K	DEAV
3001	FV	EAE	E	DEAV

From 11th International Histocompatibility Workshop and Conference, Volume 1, p.615-618.

4.2.2 Spleen Dose Response of Immunizations with Raji Cells

In the following experiments the purpose was to determine the dose of Raji for the fusion experiments to follow. Since the aim of this section was to enhance the production of hybridomas specific to Class II HLA determinants, it was decided to maximize all aspects of the experiment. In other words, choosing the best dosage of antigen to elicit a maximum primary response.

In order to accomplish this, groups of 2, eleven week old female C3H mice were injected with varying amount of Raji cells intravenously; 1.5×10^7, 3.0×10^7, 5.0×10^7, 6.0×10^7 and 7.0×10^7 cells/animal. A control group receiving only sterile PBS was also included. Injections of 7.5×10^7 cells were performed twice but resulted in the animals dying almost immediately after injection in both cases. When administering the 7.5×10^7 cell dose, heparin injected with the cells did improve the success of the injection (also seen in the administration with the higher dose of DP transfectants). However, since the effects of heparin are not completely understood (Ihrcke *et al.* 1993), it was decided not to unnecessarily complicate the experiment with an unknown factor and no experiments were performed with a 7.5×10^7 group. The spleens were removed at day 3, post injection, and the total cell count performed.

As can be seen from Figure 13, the spleen size increased as the dose of cells increased up to a peak at around a dose of 6.0×10^7 cells. Based on these data, it was

decided that an immunization dose of 6.0×10^7 cells per mouse would be used for the fusion experiments to follow. This would allow for a strong response as seen by the total spleen count, while at the same time administering a dose at a level that would allow the animals to remain in good health until the completion of the experiment.

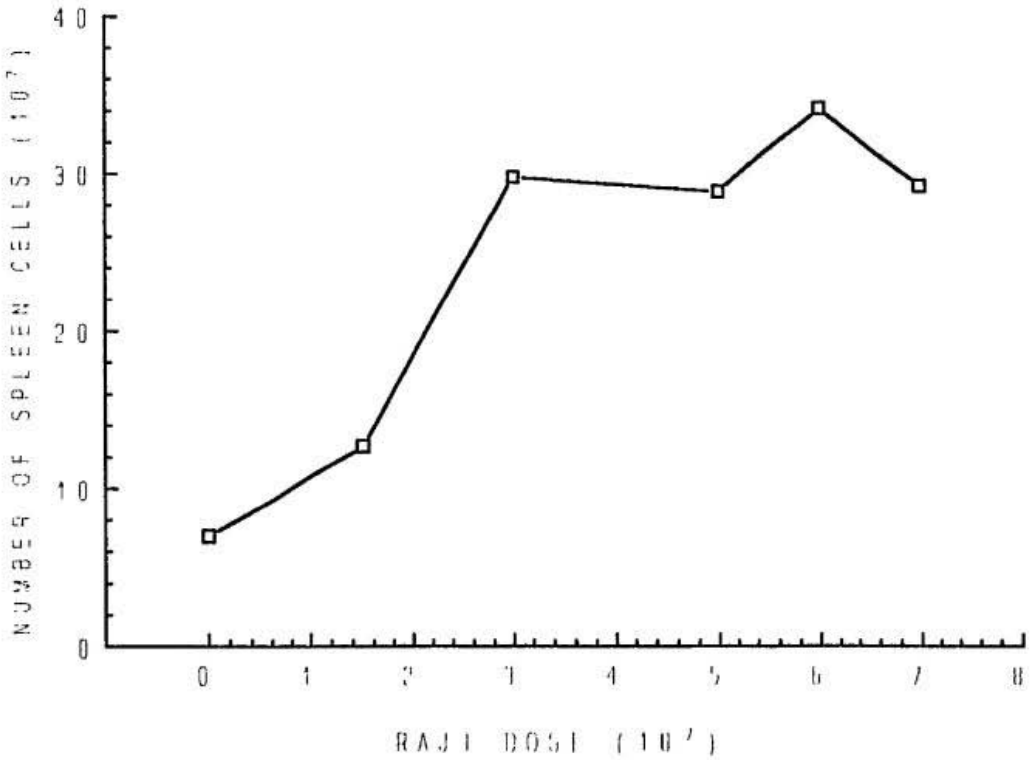

Figure 13 : Spleen size in relation to Raji dose. 11 week old female C3H mice were used. Each treatment group consisted of 2 mice. Counts represent total spleen cell number. The 0 dose represents an administration of sterile PBS.

4.2.3 Fusion Experiments

These experiments investigated the effect of differing amounts of a preexisting HLA-DP specific IgM antibody (NFLD.M65C3) on the production of hybridomas specific to Class II HLA, especially HLA-DR and HLA-DP. Throughout the results these antibody producing hybridomas are termed "hybrids/hybridomas of interest". These "hybridomas of interest" are against HLA-DR and HLA-DP molecules. This laboratory is particularly interested in obtaining hybridomas directed against; DPβ1 69(K), DPβ1 55-57(AAE/EAE) and DR10, as previously mentioned.

With the preliminary work completed, the fusion experiments were performed. Based on previous work in this laboratory, spleens were harvested at day 3 after antigen administration. Prior to the fusion experiments, C3H mice had been used for the studies reported in this thesis. F1 hybrid mice were chosen for the fusion experiments. The F1 hybrid mice were obtained from a cross between Balb/c and C3H mice. Previous experiments in this laboratory had indicated that the F1 hybrid mice produced hybridomas with a wider variety of specificities as compared to the C3H mouse strain. Thus, the F1 hybrid was used to maximize the resultant yield of hybridomas to HLA-DP and DLA-DR molecules. The F1 hybrid mice had not been used in the spleen size experiments (Figure 13) due to a lack of availability.

The design of the experiment called for three groups of 4 F1 mice. Within each group, three of the four mice were pretreated with purified antibody while the fourth mouse acted as the control receiving sterile PBS. Mice were first injected intravenously with antibody and, within 1-2 hours, injected intravenously with antigen, 6.0×10^7 Raji cells. The three treatment groups received : 2.63 mg/mouse, 1.31 mg/mouse and 0.47 mg/mouse of purified HLA-DP specific IgM antibody (NFLD.M65C3). The animals were then sacrificed on day three and the spleens aseptically removed. The spleen cells were then fused with the myeloma cell line Sp2/O. The resulting hybrids were plated out in a suitable dilution such that each well would contain approximately one hybrid cell. The hybrids were allowed to grow and were then screened against a number of cells with known HLA types so as to determine their specificity.

4.2.3.1 Spleen Size at Day 3

When the spleens were removed from the animals, counts were performed to determine the total spleen size before and after T cell depletion. These figures are shown in Table 12. 1.3×10^5 spleen cells/well were plated. This fusion procedure has been established in this laboratory with the afore mentioned cell per well number found to give optimum clonal growth of fused cells. It has been observed in previous fusions, in this laboratory, that the B cell T cell ratio is roughly 50:50. Thus, after the magnetic-bead T cell depletion method, there should be approximately 50% recovery, for example, in Table 12 the percent recovery after T cell depletion should be approximately 50%

(assuming that the B cell T cell ratio is 1:1). The control group displayed 38.4% recovery. All the IgM treated animals had a greater recovery as compared to the control group, 89.1% for the 0.47mg IgM, 45.2% for the 1.31mg IgM and 40% for the 2.63mg IgM, thus suggesting that the spleens did not consist of the 50:50 B cell T cell ratio as had been expected.

The data also suggest that the more IgM antibody administered, the more T cells there are in the spleen and conversely, the lower the IgM dose, the more B cells present in the spleen. It makes intuitive sense that the more B cells in the spleen, the more hybridomas and the better chance of obtaining the desired specificities.

Table 12 : Percentage recovery of spleen cells after T cell depletion prior to fusion with the Sp2/0 partner cell.

TREATMENT GROUP	TOTAL SPLEEN COUNT ($\times 10^8$)	AFTER T CELL DEPLETION ($\times 10^8$)	PERCENT RECOVERY OF NON - T CELLS
0.47 mg IgM	4.6	4.1	89.1%
1.31 mg IgM	5.6	2.5	45.2%
2.63 mg IgM	6.5	2.6	40.0%
CONTROL	1.9	0.73	38.4%

Spleens from IgM treated mice (3 mice per group) were pooled and T cell depleted. Groups of three mice had been pretreated with 0.47, 1.31 or 2.63 mg of IgM antibody and 1-2 hours later immunized with 6×10^7 Raji cells/animal. Spleens from each IgM treated group (3 mice) were pooled and T cell depleted. Control mice were pretreated with PBS, then immunized with 6×10^7 Raji cells/animal. The control spleens were not pooled and represent an average number. The total spleen cells after T cell depletion were fused. After fusion the cells were plated at 1.3×10^5 cells/well.

4.2.3.2 Primary Screen : Hybrid cells against Raji

At approximately day 10, the supernatants of the hybrids were considered to be rich enough to undergo the first screening procedures. This time was selected based on past fusion experiments conducted in this laboratory. The screen was carried out by CELISA with each of the hybrid supernatants being tested against the immunizing cell, Raji. This allowed for selection of all hybridomas that were specific for the immunizing cell line.

All hybrids having an absorbance reading (490nm) of greater or equal to 1.0 were considered positive and kept for future screening. The average number of viable hybrids was estimated by counting plates at random (columns 3, 7 and 11) and noting the number of viable hybrids. Plates counted for each fusion were as follows :

A. 0.47 mg IgM group : 13/26 plates were counted.
Control : 4 /4 plates were counted.

B. 1.31 mg IgM group : 10/19 plates were counted.
Control : 7/7 plates were counted.

C. 2.63 mg IgM group : 11/20 plates were counted.
Control : 5/5 plates were counted.

The percentages of immunogen reactive hybrids were calculated by determining the number of positive hybrids as seen in the primary screen and dividing by the result of multiplying the average number of hybrids per plate and the number of plates for each fusion group.

Table 13 shows the number of immunogen reactive hybrids for the different fusions as a result of the primary screen. The control animals were grouped together for analysis. Treatment groups of 1.31 mg and 2.63 mg of IgM antibody gave a lower number of immunogen reactive hybrids (9.3 and 17.7, respectively) as compared to the control group (29.3). However, the low dose of 0.47 mg antibody produced a higher number of immunogen reactive hybrids (101) as compared to the control group. Thus, at this point the data suggest that pretreatment with a low dose of IgM antibody (0.47 mg) may enhance the number of antigen specific hybridomas (as seen in the primary screen), whereas the higher IgM doses are associated with an unexpected reduction of specific hybridomas.

Table 13 : Percent positive immunogen reactive hybrids as seen in the primary screen.

TREATMENT GROUPS	AVG. # VIABLE HYBRIDS (PER SPLEEN)	AVG. # OF POSITIVE HYBRIDS (PER SPLEEN)	# OF POSITIVE HYBRIDS (%)
0.47 mg IgM	123.9	101	81.5%
1.31 mg IgM	92.5	9.3	10.1%
2.63 mg IgM	96.7	17.7	18.3%
CONTROL	63.6	29.3	46.1%

The average number of viable hybrids per spleen was estimated/calculated by counting plates at random. Columns 3, 7 and 11 of each plate were scored for the number of wells with viable hybrids. The number of immunogen positive hybrids was determined by CELISA, these hybrids reacting positively against Raji. Percentage immunogen reactive hybrids were calculated by :

$$\% \text{ Positive hybrids} = \frac{\text{Avg. \# of Positive Hybrids per Spleen}}{\text{Avg. \# Hybrids/plate} \times \text{\# of Plates for each Fusion}} \times 100$$

4.2.3.3 Secondary Screen

The secondary screen involved a negative selection process. All hybrids were tested within 24 hours of the primary screen. The cell lines used were : LBF, WT47, TOK and SAVC. These cells were pooled and tested by CELISA (i.e all cells were placed in the same wells). Table 2 gives the HLA types of these cells. The hybrids were also tested separately, against Raji (by CELISA) to confirm that the hybrids were secreting anti-Raji antibodies (immunogen specific). Hybrids were chosen for further screening if they were negative against the cell panel and positive against the immunizing cell. This selection allowed for a rapid method of eliminating hybrids with specificities that our lab was not interested in, and in doing so the large number of hybrids was dramatically reduced to a manageable number. To accomplish this, a cell panel containing such HLA types was used (Table 2). This selection was based on a percentage positive report. Cells giving greater or equal to 40% positive against Raji and less than or equal to 10% positive against the cell mixture were chosen. Thus, if the hybridomas supernatants did not react with the chosen panel and did react with Raji, those hybridomas went on for further screening.

As can be seen in Table 14, in the treatment group of 1.31 mg IgM antibody, a greater percentage of hybrids (71.4%) of interest resulted from the IgM treated animals as compared to their controls (38.6%). In the case of the 0.47 and 2.63 mg IgM group, the control group produced more hybrids of interest, (25.0% and 37.7% respectively, as

compared to the control 38.6%). Percent positive was calculated by dividing the number of hybrids of interest by the total number of hybrids screened.

Thus, upon comparison/elimination of the hybridomas with the HLA types of the cell panel, the specificity of the hybridomas continuing on to the tertiary screen, with respect to the immunizing cell can be proposed as possibly being : Class I : A 23, B 8, C ? (not 5,6,7) ; Class II : DR 3 or 10, DQ (eliminated), DP α02, β ? (not 1701, 1601, 0901, 1001).

Table 14 : Percentage of positive hybrids as seen in the secondary screen.

TREATMENT GROUPS	AVG. # HYBRIDS OF INTEREST POST SCREEN (PER SPLEEN)	AVG. # OF HYBRIDS SCREENED IN SCREEN #2 (PER SPLEEN)	PERCENT POSITIVE
0.47 mg IgM	25.3	101	25.0%
1.31 mg IgM	6.7	9.3	71.4%
2.63 mg IgM	6.7	17.7	37.7%
CONTROL	11.3	29.3	38.6%

The hybrids of interest were determined by a negative selection process against a pool of cell lines, LBF, WT47, TOK and SAVC while reacting positively with Raji in a CELISA. Percent positive was calculated by dividing the number of hybrids of interest by the total number of hybrids screened.

4.2.3.4 Tertiary Screen

The tertiary screen involved another panel of cells in a further attempt to narrow down the selected hybrids towards HLA-DR or HLA-DP specificities by CELISA. The cell lines used in this screen included : SAVC, COX, KAS116, YAR, SLE005, 8304 and Raji. The 8304 cell line is a mouse L cell transfected with human HLA-DP. Table 3 gives the HLA types of these cells.

Table 4 gives the reaction patterns of the hybrids that were selected for further screening with their (possible) respective specificities. From this selection, hybrids having possible specificities for HLA- DP, particularly DPβ1: 69 - K, 55-57 - AAE and/or EAE and HLA-DR10 were selected.

Table 14 shows the hybrids of interest resulting from the tertiary screen. The control group gave a higher percentage of hybrids of interest (55.8%) as compared to the 1.31 and 2.63 mg IgM treated group (5.0% and 15.0% respectively). However, the 0.47 mg group gave a higher percentage of hybrids of interest (67.7%) as compared to the control (55.8%). When looking more closely at the control group, the resulting 19 hybrids of interest of the 34 hybrids screened originated from the same mouse.

Two distinct reaction patterns emerged from this screen; either the hybrids of

interest were positive on Raji and negative on the remaining cells (indicating a possible HLA-DR10 antibody) or the hybrids were positive on Raji and 8304 (indicating a possible HLA-DP antibody), (8304 is a mouse fibroblast cell line transfected with human HLA-DP, expressing α02 and β0401, Table 4). The majority of the hybrids selected fitted the HLA-DR10 pattern. Of the 74 hybrids selected 68 (91.9%) fitted the DR10 and 6 (8.1%) the HLA-DP pattern. Furthermore, of the hybrids selected that indicated a HLA-DR10 specificity, 51 were produced from mice pretreated with IgM antibody (the majority of hybrids resulting from the 0.47 mg IgM dosage - 48), with the remaining 17 hybrids originating from the control group. When looking at the possible HLA-DP antibodies, the IgM treated animals produced twice as many antibodies as compared to the control group, 4 HLA-DP resulting from the 0.47 mg treatment of antibody and 2 from the control group.

Thus, at the end of the tertiary screen, due to the consistency of the reaction patterns of the hybrids, it was possible to eliminate certain specificities to which the hybrids were not directed. All the selected hybrids had failed to react with the following cell lines : LBF, WT47, TOK, SAVC, SLE005, COX or KAS116 in CELISA screenings. Therefore, with respect to the immunizing cells HLA type, the hybrids selected so far may be directed against HLA Class I : A 23, B (eliminated), C ? (not 5, 6, 7); with respect to HLA Class II : DR 10, DP α02, β ? (not 0301, 0401, 0901, 1001, 1301, 1601, 1701).

Table 15 : Percentage positive hybrids as seen after the tertiary screen.

TREATMENT GROUPS	# HYBRIDS OF INTEREST	TOTAL HYBRIDS SCREENED	PERCENT POSITIVE
0.47 mg IgM	51	76	67.7%
1.31 mg IgM	1	20	5.0%
2.63 mg IgM	3	20	15.0%
CONTROL	19	34	55.8%

The hybrids of interest were determined from Table 4 which gives the reaction patterns of the hybrids that were selected with their (possible) respective specificities. Percent positive was calculated by dividing the number of hybrids of interest by the total number of hybrids screened.

4.2.3.5 Quaternary Screen

The fourth screen involved CELISA testing against a further 6 cell lines : DUCAF, BA1088, HAS15, VAVY, TAB089, WT51 and Raji. BA1088 and Raji were the only non-homozygous cells. BA1088 (B cell) was produced in this laboratory by Epstein Barr virus transformation; the cell line originated from a person typed as having DR10 HLA type. The panel of cells allowed for a greater examination of specificity testing on the hybrids. Table 5 gives the HLA types of these cells.

Thus, with respect to the immunizing cells HLA type, the hybrids selected after this screen may be directed against HLA Class I : A (ruled out), B (ruled out), C ? (not 5, 6, 7, 8, 11); with respect to HLA Class II : DR 10, DQ (ruled out), DP α02, β ? (not 0101, 02012, 0202, 0301, 0401, 0501, 0901, 1001, 10202, 1301, 1601, 1701). At this level, hybridomas reacting with the BA1088 and Raji cell line were defined as a possible anti-HLA-DR10, and those reacting only with Raji indicating a possible anti-HLA-DP (Table 5). Past reaction patterns with all the various cells were also compared in order to ensure these designations.

Table 15 gives the number of hybrids of interest resulting from the quaternary screen. Within the 0.47 mg group, the IgM treated animals gave a higher percentage (45.1%) of hybrids of interest as compared to the control group (36.8%). The 1.31 mg IgM treatment group did not yield any hybrids of interest. The 2.63 mg treatment

group produced an equal number of hybrids of interest as compared to the control group, 33.3% vs. 36.8%. Again, one mouse within the control group produced the 7 hybrids of interest with no contribution from the two remaining control animals. The 0.47 mg treatment group produced a relatively equal number of hybrids of interest within the test group.

From comparisons with all the cells tested against the selected hybrids, the IgM treated mice produced 21 anti-DR10 and 2 anti-HLA-DP antibodies. From the controls there were 7 anti-DR10 antibodies created. When looking at the original number of hybrids created, as determined by the primary screen, a comparison of the percentage of hybrids finally selected at the end of the fourth screen can be made. From the 0.47 mg treatment group the pretreated IgM animals produced 23 hybrids secreting antibody against Class II HLA-DR and HLA-DP, 23/101 or 22.8%. The control group produced only anti-HLA-DR antibodies, 7/88 a yield of 8.0%. The 1.31 mg group did not yield any hybrids of interest. The 2.63 mg pretreated IgM animals produced 1 Class II HLA-DP antibody 1/53 or 1.9%. Animals given the lower dosage of IgM antibody, 0.47 mg, did seem to have a better overall yield of antibody formation to Class II MHC determinants as compared to the 1.31 mg and 2.63 mg groups with the specificity predominately directed against HLA-DR10 molecules.

Table 16 : Percentage positive hybrids as seen in the quaternary screen.

TREATMENT GROUPS	# HYBRIDS OF INTEREST	TOTAL HYBRIDS SCREENED	PERCENT POSITIVE
0.47 mg IgM	23	51	45.1%
1.31 mg IgM	---	1	---
2.63 mg IgM	1	3	33.3%
CONTROL	7	19	36.8%

CHAPTER 5

DISCUSSION

Although the experiments reported here have confirmed in a general way the earlier published results, namely that pretreatment with antigen specific IgM antibody facilitates an enhanced primary response and conversely the pretreatment with antigen specific IgG antibody mediates a suppressed response, there were several unexpected findings. These include a reverse dose response when transfectant cells were used as an immunogen; an apparent reduction of the IgM enhancement when higher doses of IgM were used; a curious effect on the half life of IgM *in vivo*, where high doses seemed to lead to a lengthening of the half life and finally an interesting effect of specific IgM on the number of T cells in the subsequently immunised spleen. These points will be discussed in turn which will be followed by an attempt to understand the complex data collected during the fusion experiments. In a last section a plan for future research is presented.

5.1 *In Vivo* Experiments With Mouse Transfectant and Human B Cell Lines

In the *in vivo* experiments, certain protocols were adapted from studies reviewed in the Introduction. Modifications made to the experimental protocol were time of antigen injection (1-2 hours post antibody injection), route of injection (I/V)

and amount of antibody administered. All these variables were previously optimized in their respective experiments, with the findings adapted to the experiments designed in this thesis project.

5.1.1 Reverse Dose Response With Transfectants

Interestingly, the two immunogens, L25.4 and Raji, displayed differing dose responses (Figure 8). The human B cell line, Raji gave the expected dose response with increasing dosage increasing the response, whereas the L cell transfectant line, L25.4, gave an inverted response. A dose of 5×10^6 L25.4 cells/animal gave the highest antibody response and, increasing the dosage to 1×10^7 and then 2.5×10^7 cells/animal, actually resulted in a progressively lower production of antibody. The most likely explanation for this is that the higher cell doses may be actually inducing a degree of tolerance. Such a "high dose tolerance" effect is well recognized especially for weak antigens (Matzinger, 1994). Since L25.4 is a transfected mouse fibroblast expressing Human HLA-DP molecules (originating form a C3H mouse), when these cells are injected into a C3H mouse, the animal will see only the expressed Human HLA-DP antigens as foreign. In contrast, the Raji cell line is a human B cell lymphoma. When presented in a mouse, the animal will recognize the entire repertoire of expressed human antigens as foreign and subsequently a vigorous response will be mounted. Thus, the Raji cells are a much stronger antigen than the L25.4 cells. The finding that it may be difficult to induce tolerance with strong

immunogens (Matzinger, 1994) also provides supporting evidence for the discrepancy between immunogens. This B cell tolerisation may also be a result of the route of injection (intravenously) and/or the high dosage of antigen administered (Nossal, 1992; reviewed in Abbas *et al.* 1991). The experiments in this study showed that the Raji cell line did not demonstrate any reduction of the response even with an administration of 2 x 10^7 cells/animal (intravenously). Further experiments are worth conducting. To initiate an investigation of this phenomenon, cell dose experiments could be performed starting at quite low doses of 1 x 10^5 L25.4 cells with gradual increments to 1 x 10^8 cells/animal. As always, it would essential that enough mice be used in the groups to allow the data to be statistically significant. It would also be interesting to see if cells such as L556 (mouse fibroblast transfected with human HLA-DR) also demonstrate a tolerisation effect at high immunizations.

5.1.2 *In Vivo* Experiments with Raji as Antigen

The *in vivo* experiments were first conducted with Raji cells as the immunogen. This experiment showed that animals pretreated with specific IgM antibody displayed a significantly enhanced response as compared to the controls (Figure 9). Sera were collected on day 7 and tested separately with IgM and IgG specific conjugates to determine respective changes in primary antibody levels. Animals treated with specific IgM antibody displayed a statistically significant enhancement of the serum antibody directed against Raji with respect to both IgM and IgG levels as compared to the PBS

control. This confirms experiments in the literature in which red blood cells were used as antigen and showing IgM enhancement (Heyman,1990b). Conversely, animals treated with specific IgG antibody displayed a diminished anti-Raji IgM and IgG response as compared to the PBS control. However, in this case, when statistical analysis was employed the differences were not significant. This may be due to the IgG antibody being administered at a sub-optimum dose. To test this, a similar experiment should be conducted in which a range of specific IgG antibody is administered in order to identify this optimum dosage for the IgG suppression. This would also be worth performing for the IgM mediated enhancement. Consideration should also be given to whether the IgG suppression effect is influenced or not by the antibody purification protocol.

5.1.3 *In Vivo* Experiments with L25.4 Cells as Antigen

5.1.3A Initial Experiment

The first experiment, conducted with L25.4 as antigen, not only displayed a tremendous enhancement facilitated by the pretreatment with specific IgM antibody but also demonstrated a suppressed response mediated by specific IgG antibody upon comparison with the controls (Figure 10). Both the observed enhancement and suppression were statistically significant. This again confirms experiments in the literature in which red blood cells used as antigen showed specific IgM mediated

enhancement and specific IgG mediated suppression (Heyman, 1990b). Thus, this phenomenon, earlier described by Henry and Jerne (1968) has been established to exist when the antigen used is nucleated cells, namely a human B cell lymphoma; Raji and a mouse fibroblast transfected with human HLA-DP; L25.4.

5.1.3.B Diminished Response with Doubling of Antibody

An unsettling observation was made in the second set of experiments with the L25.4 cell line (Figure 11). In this experiment treatment groups consisted of one receiving specific IgM antibody, another receiving non-specific antibody and a control group identical to the previous experiments. The previously demonstrated enhancement mediated by specific IgM antibody diminished drastically in this experiment. The 'non-specific IgM' antibody actually appeared to enhance the subsequent formation of antibody, rather then have no affect as had been anticipated. Upon further comparison to the previous experiment with L25.4 (Figure 10), an additional factor was altered, that being the administered antibody dosage.

5.1.3.B1 Antibody Dosage

In comparison to the previous experiment (Figure 10), in an attempt to improve the enhanced response of the IgM antibody, larger doses of both the specific and the non-specific IgM antibodies were administered. This alteration was based on the

observation made from the experiments of Henry and Jerne (1968) who clearly showed that there exists a direct linear relationship between the degree of enhancement and the administered dose of the IgM antibody as demonstrated by red cell antigens and anti-red cell antibodies with plaque forming assays. However, some researchers have shown, in contradiction to these experiments, that there is an upper limit to the effect of IgM antibody pretreatment, beyond which the response ceases to display the characteristic enhancement (Dennert,1971; Powel *et al.* 1982) .

One possible explanation for the results in Figure 11 is that the 'upper limit' may have been passed in this experiment. It would be interesting to pinpoint the optimal dosage of administered antibody. A range of doses for pretreatment with IgM, IgG and the non-specific IgM antibody could be examined, for example, from 0.1 mg to 1.0 mg of antibody per mouse in 0.1 mg increments. The experimental design would consist of treatment groups of at least 4 mice per group. The control group would not receive any antibody treatment. The animals would be bled prior to any manipulation so as to detect existing IgG and IgM antibody levels. The animals would then be injected with the appropriate antibody and 1-2 hours later with antigen. The animals could then be bled every third day for twelve days and the serum assayed.

Another concern comes from the rate of decay/elimination of the administered antibody. The high level of detected antibody in circulation within this experiment (Figure 11), as compared to the previous one (Figure 10), suggests that there is a

limit to the amount of antibody getting to the spleen. This limit may have an important role in the IgM and IgG mediated modulations. It would be interesting to investigate if tissues other than the spleen also take up the administered IgM antibody. If this is the case, the method of capture/uptake would also be interesting to know. If other tissues are not found to be involved, how is this excess antibody and/or antibody-antigen complexes destroyed. A preliminary investigation into these queries could be initiated by designing an experiment in which different groups of mice receive a broad range of IgM antibody. Serum samples would then be taken at consistent time intervals. Duplicate and/or multiple groups, for the varying doses, would allow for obtaining tissue samples at the different times. Both serum and tissue samples would then be assayed for the presence of the administered antibody.

5.1.3.B2 Non-specific IgM

A review of the IgM enhancement phenomenon states that the antibody used for pretreatment must be antigen specific but not epitope specific (Heyman 1990b). To test this, a non-specific IgM antibody was used. This antibody was chosen based on previous Flow Cytometry analysis (Figure 5) and CELISA results (Table 7) both demonstrating extremely low binding with L25.4. According to the cited literature, it was expected that this non-specific IgM antibody would have no enhancing capacity in this experiment. This was not the case since the non-specific antibody treated group actually mediated a small degree of enhancement as compared to the controls. Thus,

the specificity of the 'non-specific' IgM antibody can be questioned. It may be possible that there was some small degree of specificity or unanticipated crossreactivity with this antibody, and this may provide an explanation of the observed slight enhancement. Selecting a non-specific IgM antibody for further experiments would involve testing a panel of IgM 'non-specifics' against a number of different cell lines with known specificities. This would allow for an extensive demonstration of specificity.

5.1.3.B3 Actual Amount of Antibody and Antigen Received

Another factor to be considered in this second set of experiments with L25.4 (Figure 11), is the actual amount of administered antibody and antigen the animals received upon injection. Since intravenous administration of antibody and antigen was particularly difficult due to the size of the tail veins, records were kept as to the 'success' of injections for all experiments. These records indicated that the administration of both antibody and antigen was not ideal for all the animals in this experiment. This problem must be considered when interpreting the results. If this experiment is to be repeated, as with any of the *in vivo* experiments, it would be convenient to have extra animals, antibody and antigen on hand when performing the injections. This would allow the investigator to select mice with a smaller margin of error in the actual amount of antibody and antigen received by the animals, while at the same time maintaining a treatment group number that is statistically significant.

Thus, the above described experiments demonstrated that the enhancement/suppression phenomenon described by Claudia Henry and Niels Jerne (1968) is also observable with nucleated antigens. With this observation made, the application of this phenomenon to the creation of hybridomas was then investigated.

5.2 Fusion Experiments

For the fusion experiments F1 hybrid mice were utilized, while the *in vivo* experiments employed C3H mice as did the preliminary spleen dose response experiment (Figure 13). The spleen dose response experiment utilized C3H only due to the lack of availability of the F1 hybrid mice. In an effort to maximize specificity, variability and viability of the hybridomas, this laboratory has used F1 hybrid mice. The F1 hybrid mice contain a heterozygous array of MHC molecules, originating from Balb/c and C3H parents. It was hoped that this heterozygosity would also lead to more variability in the anticipated increased cell response. The fusion partner cell for the F1 hybrid B cells was the Sp2/O cell line. This cell line originated from a Balb/c mouse. The second rationale for using F1 hybrid mice, was to minimize any B cell (experimental animal) anti-fusion partner cell response that might develop. Previous experiments conducted in this laboratory indicated that these hybrid mice produced a wider variety of specificities as compared to the homozygous mouse strains used (for example Balb/c, C3H).

5.2.1 Choice of Antibody

The criterion for the specific IgM antibody used for pretreatment is that it had to have strong binding to the antigen of choice. The antibody need not be epitope specific but had to be antigen specific. The IgM antibody used was specific for a monomorphic determinant of HLA-DP. This antibody had already been shown to have strong binding with Raji (Figure 5) and had demonstrated a strong enhancement when antibody was used to pretreat animals prior to injection of the Raji cell line (Figures 9 and 10). The proposed *in vivo* mechanism of enhancement is that the effective trapping/localization of antigen within the spleen is facilitated by specific IgM antibody (see Introduction). On hindsight, if the specific IgM antibody is acting in such a manner, with the antibody binding to HLA-DP epitopes, it may have been better to have used an antibody against monomorphic HLA-DQ or other determinants which efficiently bound to Raji, but which may have left the HLA-DP and HLA-DR specific epitopes more exposed to be recognized or bound by B cells in the spleen. This may have a resulted in a more productive yield of HLA-DP and DR antibodies. At a purely speculative level, this explanation may also account for the predominance of HLA-DR specific hybridomas observed at the fourth screen as a result of the use of the HLA-DP specific IgM antibody.

5.2.2 Spleen Analysis

5.2.2A Spleen Size and Dose of Antibody

There was an effect on spleen size of increasing doses of specific IgM antibody. As the dose of IgM was increased from 0.47 mg to 1.31 mg to 2.63 mg per mouse the number of cells of the spleen rose concomitantly from 4.6×10^8 to 5.6×10^8 to 6.5×10^8. Several points need comment here.

First, the spleen size went above the previous plateau demonstrated by increasing doses of Raji cells. This may be a result of a particular mouse strain effect, F1 versus C3H mice and will need further exploration by a direct comparison of the F1 and C3H mice. One should first investigate if a plateau effect exists, as described with C3H mice, with increasing doses of Raji cells. Following this, one would investigate varying doses of specific IgM antibody in groups of F1 and C3H mice. From these results, with proper controls and sufficiently large numbers of animals, a comparison of the two strains would be possible. It would be established if specific IgM really does lead to an increase in spleen size in relation to antigen dose and/or if the "maximum size" can be exceeded.

5.2.2B Spleen T Cell Composition

Second, although the spleen cell number was increased, it was apparently mainly due to an increase in T cells. The number of B cells, after T cell depletion,

decreased with increasing IgM doses. This curious observation has evidently not been made before. Further, investigation may identify the type of T cell population found in the spleens. One can speculate that the T cell population is composed of mainly $CD8^+$ cells, with the real possibility that some type of suppressive mechanism is involved. If by T cell population analysis there indeed is a predominance of $CD8^+$ T cells, this could be investigated by conducting an experiment in which the spleens are depleted of their $CD8^+$ population and the original experiment repeated. This may provide a way of further enhancing the response. In this situation, it follows that the B cell population would be increased and thus, the final result might be an increased number of hybridomas with varied specificities.

5.2.3 Specificity Analysis

5.2.3.1 The Screening Rationale

The ultimate goal of the screening strategies was to identify and select certain the specificities of the hybridomas created after the fusion. Due to the enormously large number of hybridomas created it was necessary to develop a strategy which facilitated the rapid elimination of hybridomas that were not of interest. The hybridomas of interest for this study were defined before the onset of these experiments as being HLA-DR and HLA-DP specific, particularly those directed against DPβ1 69(K), DPβ1 55-57(AAE/EAE) and DR10.

The first screen was conducted by CELISA in which the immunizing cell, Raji, was tested against the supernatants from all the viable hybridomas created. This immediately selected for all the hybridomas that were antigen specific. As can be seen from Table 13, there was a marked reduction in number of hybrids initially screened (1130) and the number of positive hybrids selected (472). The selected hybrids then were tested with a series of screening panels of homozygous cell lines. This made possible the rapid elimination of hybridomas and elucidation of specificities of those remaining.

The second screen was a negative selection process. The selected hybridomas were tested against a panel of homozygous cell lines (Table 14) all of which had HLA specificities that were of no interest. Thus, hybridomas whose supernatants bound with Raji cells and did not bind with the panel, were chosen to be carried forward to the next screening. A concern here is that this screen eliminated many types of anti-Raji hybridomas, including those against Class I polymorphisms and monomorphic Class II determinants of all kinds. Theoretically, the IgM facilitated enhancement is 'particle dependent' (see Introduction) and thus, all anti-Raji specificities should be increased. Although many specificities were eliminated through the selection process, the selected specificities of interest should also be increased and this should be demonstrated by direct comparison between treatment groups, a reference/control group, all of which had undergone the same selection criteria.

For the tertiary screen a reaction pattern was developed that would indicate the possible specificities of the screened hybridomas (Table 4). In this way, the hybridomas specificities were further narrowed and the number of hybridomas reduced. At this level, all of the selected hybridomas displayed a reaction pattern suggesting either a possible anti-DR or anti-DP specificity. This may lead one to suspect the possibility of the pretreatment of specific IgM to specifically direct a response. At this stage this is purely speculative and would need further study to verify. The fourth screen used yet another panel of homozygous cell line, further elucidating the hybridomas specificity. The panel contained a heterozygous cell line (BA1088) which expressed the HLA-DR10 antigen (Table 5). Hybridomas reacting with the BA1088 and Raji cell line were defined as a possible anti-HLA-DR10, and those reacting only with Raji indicating a possible anti-HLA-DP (Table 5). Past reaction patterns with all the various cells were reanalyzed in order to ensure these designations.

The cell line panel chosen was composed of lines that were not problematic to grow and that were available in this laboratory. A larger panel of cell lines would have provided better specificity definition if readily available.

5.2.3.2 Specificity of Resultant Hybridomas

In the fusion experiments, three doses of IgM antibody (0.47mg/mouse,

1.13mg/mouse and 2.63mg/mouse) were administered to three groups of mice, all containing control animals. This was done in order to test whether antibody pretreatment dose affected the number or variety of hybridomas created (particularly those with specificities deemed of interest). When 0.34 mg/mouse of specific IgM antibody was administered the enhancement was clearly significant, however, when the dosage was doubled to 0.66 mg/mouse (Figure 11) it was not. Thus, dosage of IgM antibody may have an important role to play in the enhancement phenomenon. One must be careful in adapting this finding to fusion experiments, since maximum localization of antigen in the spleen by the pretreatment IgM antibody may be important in the development of monoclonals to particular epitopes of interest (especially those that have been problematic), than the quest of solely enhancing the response. In other words the antigen must be trapped, facilitating maximum presentation to the B cells.

The experiments suggest but do not prove that treatment with low dose of specific IgM increases the yield of specific hybridomas.. Animals pretreated with 0.47mg IgM antibody gave a 22.8% yield of HLA-DR and DP monoclonals, whereas the control group produced an 8% yield of only HLA-DR monoclonals. Compared to the other IgM dosages, the 0.47 mg dose contributed 96% of the total HLA-DP/DR selected monoclonals. Although there was much individual variation in control animals, nevertheless, on a percentage basis, the IgM treated animals clearly produced more anti HLA-DP and HLA-DR hybrids. For future experiments it will be important

to use far more animals in order to accurately determine the effect of the specific IgM antibody. In addition, animals in the treatment group should be fused separately, rather than pooled before fusion, in order to compare individual variation in these groups with those of control animals.

5.3 Future Directions

These pilot studies of the enhancement/suppression phenomenon using nucleated cells indicate that this technique warrants further investigation. As mentioned in the Introduction, the exact mechanism(s) involved in IgM facilitated enhancement is not fully understood. Experiments to elucidate the mechanisms would involve careful planning with innovative approaches. The results, however, would be of great value not only on a pure immunological level but also for practical applications.

The role of the FcR receptors should be investigated. Since one of the postulated mechanism of IgG suppression involves crosslinking by immune complexes of FcγRIIB and antigen receptors on B cells, it may be possible to use genetically altered mice that have a FcRγ chain deletions (Ravetch,1994). An experiment might involve groups of mice, including an FcRγ "knockout" group and two corresponding groups of strain-matched, normal mice one receiving the exact treatment as the "knockout" group and one without antibody pretreatment. In this way further insight to the regulation of B cell activation and the role of FcR's might be obtained. This

type of design also could also be used to examine the role of FcRγ in IgM pretreatment.

Interestingly, in one of the earlier experiments cited, (Wiersma *et al.* 1989), it was demonstrated that with a panel of 9 IgG monoclonal antibodies, only 5 were able to suppress the response to SRBC's. This may also hold true for the DP monoclonal antibodies chosen, including the IgM antibody. Thus, future experiments should investigate a panel of specific IgG and IgM DP monoclonal antibodies to determine if such a phenomenon exists with DP and in order to select the best antibody in both cases.

The use of other specific IgM antibodies against, for example, monomorphic determinants to HLA-DQ, might be more useful for developing monoclonals to HLA-DP and/or HLA-DR epitopes. Trapping the antigen by the HLA-DQ molecules would leave the HLA-DP and HLA-DR molecules more exposed, optimizing the presentation and subsequently enhancing the formation of antibodies to epitopes that until now have proven very problematic. It would be necessary to conduct an experiment with one group of mice receiving an anti-HLA-DQ IgM specific antibody, another an anti-HLA-DP IgM antibody, and a control group receiving sterile PBS. All the mice would then be injected with the same antigen, and after fusion the resultant hybridomas screened for specificity. It then would be able to determine if an IgM antibody specific to HLA-DQ determinants is better or worse than a monomorphic HLA-DP IgM antibody

in the creation of hybridomas to HLA-DP and HLA-DR epitopes.

Although this project tested the effect of preexisting antibody levels on the primary response only, it might be possible to apply these findings to the secondary response. An experiment might involve a setup in which a sub-optimal dose of antigen is given and, after the primary response dissipates (i.e. serum antibody levels fall close to normal), the animals could then be administered specific IgM antibody and challenged with the same antigen. In this way the secondary response might be "boosted or optimized". This technology might further increase the chance of creating hybridomas specific for a varied number of epitopes to poorly immunogenic molecules (for example, HLA-DP). This method might also increase the chances of obtaining hybridomas specific to epitopes that until now have proven to be elusive.

The hybridoma production experiments performed in this thesis can be viewed as a pilot study of one method to improve conventional fusion methodology which has relied on repeated immunisations prior to fusion for the production of monoclonal antibodies.

LITERATURE CITED

Abbas,A.K., A.H.Lichtman and J.S.Pober. Cellular and Molecular Immunology. W.B.Saunders Company, USA, 1991.

Abrahams,S., Phillips,R.A. and Miller,R.G. Inhibition of the immune response by 7S antibody. (1973). J. Exp. Med. 137:870-892.

Anchylose,J.C., H.Fleet and I.S.Mailman. Structural aspects and heterogeneity of immunoglobulin Fc receptors. (1981). Ad. Immunol. 31:247-270.

Anderson,B., A.C.Skoglund and A.Rosen. Functional characterization of mouse T lymphocytes with IgM-Fc receptors. (1979). J. Immunol. 123:1936-1939.

Berridge,M.J. Inositol trisphosphate and diacylglycerol as second messengers. (1984). Biochem. J. 220:345-360.

Bindon,C.I., G.Hale and H.Waldmann. Importance of antigen specificity for complement-mediated lysis by monoclonal antibodies. (1988). Eur. J. Immunol. 18:1507-1514.

Bijsterbosch,M.K and G.G.B.Klaus. Crosslinking of surface immunoglobulin and Fc receptors on B lymphocytes inhibits stimulation of inositol phospholipid breakdown via the antigen receptors. (1985). J. Exp. Med. 162:1825-1836.

Britton,S., T.Wepsic and G.Moller. Persistence of immunogenicity of two complex antigens retained *in vivo*. (1968). Immunology 14:491-501.

Bruggeman,M and K.Rajewsky. Regulation of the antibody response against hapten-coupled erythrocytes by monoclonal antihapten antibodies of various isotypes. (1982). Cellul. Immunol. 71:365-373.

Cambier,J.C., C.M.Pleiman and M.R.Clark. Signal transduction by the B cell antigen receptor and its coreceptors. (1994). Annu. Rev. Immunol. 12:457-486.

Carter,R.H., M.O.Spycher, Y.C.NG, R.Hoffman and D.T.Fearon. Synergistic interaction between complement receptors type 2 and membrane IgM on B lymphocytes. (1988). J. Immunol. 141:457-463.

Clarke,C.A., W.T.A.Donohoe, R.B.McConnel, J.C.Woodrow, R.Finn, J.R.Krevans, W.Kulke, D.Lehane and P.M.Sheppard. Further experimental studies on the prevention of Rh haemolytic disease. (1963). Br. Med. J. 1:979-984.

Collisson,E.W., B.Anderson, M.Ronnholm and E.W.Lamon. Potentiation of antibody

responses by specific IgM : specificity and thymus dependency. (1983). Cellular Immunology 79:44-55.

Coulie,P.G and Snick,J.V. Enhancement of IgG anti-carrier responses by IgG_2 anti-hapten antibodies in mice. (1985). Eur. J. Immunol. 15:793-798.

Dennert,G., H.Pohlit and K.Rajewsky. In Cell Interaction and Receptor Antibodies in Immune Responses. (Makela, O., A. Cross and T.V. Kosunen, eds.), p.3. Academic Press, New York, 1972.

Dennert,G. Effects of IgM on the *in vivo* and *in vitro* immune response. (1973). Proc. Soc. Exp. Biol. Med. 143:889-893.

Dennert,G. The mechanism of antibody-induced stimulation and inhibition of the immune response. (1971). J. Immunol. 106:951-955.

Ehlenberger,A.G and V.Nussenzweig. The role of membrane receptors for C3b and C3d in phagocytosis. (1977). J. Exp. Med. 145:357-371.

Egwang,T.G and A.D.Befus. The role of complement in the induction and regulation of immune response. (1984). Immunology 51:207-224.

Enriquez,R and G.G.B Klaus. Differing effects of monoclonal anti-hapten antibodies on humoral responses to soluble or particulate antigens. (1984). Immunology 52:129-136.

Erdei,A., F.Melchers, T.Schulz and M.Dierich. The action of human C3 in soluble or cross-linked form with resting and activated murine B lymphocytes. (1985). Eur. J. Immunol. 15:184.

Farkcus,A.I., G.A.Medgyesi, G.Fust, K.Miklos and J.Gergely. Immunogenicity of antigen complexed with antibody. I. Role of different isotypes. (1982). Immunology 45:483-492.

Fisch,P., G.Weil-Hillman, M.Uppenkamp, J.A.Hank, B.P.Chen, J.A.Sosman, A.Bridges, O.R.Colamonici and P.M.Sondel. Antigen-specific recognition of autologous leukaemia cells and allogenic Class-I MHC antigens by IL-2-activated cytotoxic T cells from patients with acute T-cell leukaemia. (1989). Blood 74(1):343-353.

Gergely,J., Z.Rozsnyay and G.Sarmay. New aspects of FcγRII-mediated B-cell inhibition : switch of FcγRIIb isoforms depends on B-cell activation. (1994). The Immunologist 2/3:77-86.

Gordon,J and R.A.Murgita. Suppression and augmentation of the primary *in vitro* immune response by different classes of antibodies. (1975). Cellul. Immunol. 15:392-402.

Harte,P.G., A.Cooke and J.H.L.Playfair. Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination.(1983). Nature 302: 256-258.

Heinen,E., D.Radoux, C.Kinet-Denoel, M.Moeremans, J.DeMey and L.J.Simar. Isolation of follicular dendritic cells from human tonsils and adenoids. III. Analysis of their Fc receptors. (1985). Immunology 54:777-784.

Henry,C and Jerne,N.K. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. (1968). J. Exp. Med. 128:133-152.

Heyman,B. Non-determinant specificity of feedback immunosuppression by IgG antibodies injected after the antigen. (1988). Scand. J. Immunol. 27:361-365.

Heyman,B. Inhibition of IgG-mediated immunosuppression by monoclonal anti-Fc receptor antibody. (1989). Scand. J. Immunol. 29:121-126.

Heyman,B. Fc-dependent IgG-mediated suppression of the antibody response : fact or artefact? (1990). Scand. J. Immunol. 31:601-607.

Heyman,B. The immune complex : Possible ways of regulating the antibody response. (1990b). Immunol. Today. 11:310-313.

Heyman,B., G.Andrighetto and H.Wigzell. Antigen-dependent IgM-mediated enhancement of the sheep erythrocyte response in mice. Evidence for induction of B cells with specificities other than that of the injected antibodies. (1982). J. Exp. Med. 155:994-1009.

Heyman,B and H.Wigzell. Immunoregulation by monoclonal sheep erythrocyte-specific IgG antibodies : suppression is correlated to level of antigen binding and not to isotype. (1984). J. Immunol. 132(3):1136-1143.

Heyman,B and H.Wigzell. Specific IgM enhances and IgG inhibits the induction of immunological memory in mice. (1985). Scand. J. Immunol. 21:255-266.

Heyman,B., M.Nose and W.O.Weigle. Carbohydrate chains on IgG2b : a requirement for efficient feedback immunosuppression. (1985). J. Immunol. 134(6):4018-4023.

Heyman,B., L.Pilstrom and M.J.Shulman. Complement activation is required for IgM-mediated enhancement of the antibody response. (1988). J. Exp. Med. 167:1999-2004.

Heyman,B., E.Wiersma and M,Nose. Complement activation is not required for IgG-mediated suppression of the antibody response. (1988b). Eur. J. Immunol. 18:1739-1743.

Hoffman,M.K and J.W.Kappler. Two distinct mechanisms of immune suppression by antibody. (1978). Nature 272:64-65.

Hoffmann,M.K., J.W.Kappler, J.A.Hirst and H.F.Oettgen. Regulation of the immune response. V. Antibody-mediated inhibition of T and B cell cooperation in the *in vivo* response to red cell antigens. (1974). Eur. J. Immunol. 4:282-286.

Ihrcke,N.S., L.E.Wrenshall, B.J.Lindman and J.L.Platt. Role of heparin sulfate in immune system-blood vessel interactions. (1993). Immunol. Today 14(10):500-505.

Inoko,H., J.G.Bodmer, J.M.Heyes, S.Drover, J.Trowsdale and W.H.Marshall. Joint report on the transfectant/monoclonal antibody component. In: Tsuji,K., M.Aizawa and T.Sasazuki, Eds. HLA 1991, proceedings of the eleventh international histocompatibility workshop and conference held in Yokohama, Japan, 6-13 November, 1991, Vol.1. New York: Oxford University Press; 1992:919-930.

Janeway,C.A., R.A.Murgita, F.I.Weinbaum, R.Asofsky and H.Wigzell. Evidence for an immunoglobulin-dependent antigen-specific helper T cell. (1971). Proc. Natl. Acad. Sci. USA 74(10):4582-4586.

Johnson,A.H., E.Thorsby, T.Nakatsuji, T.Fang, T.Moen and R.J.Hartzman. Recognition of an HLA-DPw1 specific alloantiserum raised by planned immunization. (1986). Human Immunology 17:21-29.

Kappler,J.W., A.VanDerHoven, U.Dharmarajan and M.Hoffmann. Regulation of the immune response. IV. Antibody-mediated suppression of the immune response to haptens and heterologous erythrocyte antigens *in vitro*. (1973). J. Immunol. 111(4):1228-1235.

Kimura,K., R.P.Dong, H.Harada and T.Sasazuki. DNA typing of HLA class II genes in B-lymphoblastoid cell lines homozygous for HLA. In: Tsuji,K., M.Aizawa and T.Sasazuki, Eds. HLA 1991, proceedings of the eleventh international histocompatibility workshop and conference held in Yokohama, Japan, 6-13 November, 1991, Vol.1. New York: Oxford University Press; 1992:422-423.

Kolsch,E., J.Oberbarnscheidt, K.Bruner and J.Heuer. The Fc-receptor : its role in the transmission of differentiation signals. (1980). Immunol. Rev. 49:62-78.

Lamon, E., B.Anderson, H.D.Whitten, M.Hurst and V.Ghanta. IgM complex receptors on subpopulations of murine lymphocytes. (1976). J. Immunol. 116:1199-

1203.

Melchers,F., A.Erdei, T.Schultz and M.P.Dierich. Growth control of activated, synchronized murine B cells by the C3d fragment of human complement. (1985). Nature 317:264-267.

Marsh,S.G.E., J.H.Moses and J.G.Bodmer. HLA class II sequence polymorphism detectable by serology. In: Tsuji,K., M.Aizawa and T.Sasazuki, Eds. HLA 1991, proceedings of the eleventh international histocompatibility workshop and conference held in Yokohama, Japan, 6-13 November, 1991, Vol.1. New York: Oxford University Press; 1992:610-620.

Matzinger, P. Tolerance, danger, and the extended family. (1994). Annual Review of Immunology 12:991-1045.

Moller,G and H.Wigzell. Antibody synthesis at the cellular level : antibody-induced suppression of 19S and 7S antibody response. (1965). J. Exp. Med. 121:969-989.

Moller,G. Antibody-mediated suppression of the immune response is determinant specific. (1985). Eur. J. Immunol. 15:409-412.

Nossal,G.J.V. Cellular and molecular mechanisms of B lymphocyte tolerance. (1992). Advances in Immunol. 52:283-331.

O'Garra,A., K.P.Rigley, M.Holman, J.B.McLaughlin and G.G.B.Klaus. B-cell-stimulatory factor 1 reverses Fc receptor-mediated inhibition of B-lymphocyte activation. (1987). Proc. Natl. Acad. Sci. USA 84:6254-6258.

Oberbarnscheidt,J and E.Kolsch. Direct blockade of antigen-reactive B lymphocytes by immune complexes. An 'off' signal for precursors of IgM-producing cells provided by the linkage of antigen and Fc-receptors. (1978). Immunology 35:151-157.

Pearlman, D.S. The influence of antibodies on immunologic responses. I. The effect on the response to particulate antigen in the rabbit. (1967). J. Exp. Med. 126:127-148.

Pepys,M.B. Studies *in vivo* of Cobra factor and murine C3. (1975). Immunology 28:369-377.

Phillips,N.E and D.C.Parker. Subclass specificity of Fc_γ receptor-mediated inhibition of mouse B cell activation. (1985). J. Immunol. 134(5) : 2835-2838.

Pollack,W., W.Q.Ascari, J.F.Crispen, R.R.O'Connor and T.Y.Ho. Studies on Rh prophylaxis. II. Rh immune prophylaxis after transfusion with Rh-positive blood. (1971). Transfusion 11(6):340-344.

Powell,R., P.Hutchings, A.Cooke and P.M.Lydyard. Antibody mediated regulation of immune responses. Enhancement of specific antibody responses through IgM antibodies. (1982). Immunol. Lett. 4:253-258.

Quintana,I.Z., A.V.Silveira and G.Moller. Regulation of the antibody response to sheep erythrocytes by monoclonal IgG antibodies. (1987). Eur. J. Immunol. 17:1343-1349.

Ravetch,J.V. Fc receptors : rubor redux. (1994). Cell 78:533-560.

Reiter,J., P.Hutchings, P.M.Lydyard and A.Cooke. Antibody-mediated modulation of the immune response. (1986). Cellular Immunol. 97:91-101.

Ryder,R.J.W and R.S.Schwartz. Immunosuppression by antibody : localization of site of action. (1969). J. Immunol. 103:907.

Samuels, L. Effect of preexisting antibody on the result of immunisation of mice with human B cells [Honours Thesis]. St. John's, Newfoundland, Canada: Memorial University of Newfoundland; 1992.

Schrader,J.W. Regulation of the immune response by IgM antibody : a paradoxical suppression of the *in vitro* primary response to sheep erythrocytes by passive IgM. (1973). Ajebak 51(3):333-346.

Schreuder, G.M.T., I.Doxiadis, J.Parlevliet and G.Wilde. HLA-DR, DQ, LB and TA10 specificities of ninth workshop homozygous typing cells. In: Albert,E.D., M.P.Baur and W.R.Mayr, eds. Histocompatibility testing 1984 : report on the ninth international histocompatibility workshop and conference held in Munich, West Germany, May 6-11,1984 and in Vienna, Austria, May 13-15,1984. New York: Springer-Verlag; 1984:243-245.

Sinclair,N.R.StC. Regulation of the immune response. I. Reduction in ability of specific antibody to inhibit long-lasting IgG immunological priming after removal of the Fc fragment. (1969). J. Exp. Med. 129:1183-1201.

Sinclair,N.R.StC. Immunoregulation by antibody and antigen-antibody complexes. (1978). Transplant. Proc. 10:349-353.

Smith,T., Active immunity produced by so-called balanced or neutral mixtures of diphtheria toxin and antitoxin. (1909). J. Expt. Med. 11:241-257.

Soos,J.M, J.K.Russell, M.A.Jarpe, C.H.Pontzer and H.M.Johnson. Identification of binding domains of the superantigen, toxic shock syndrome-1, for Class II MHC molecules. (1993). Biochem. Biophy. Res. Comm. 191(3):1211-1217.

Takai,T., M.Li, D.Sylvestre, R.Clynes and J.V.Ravetch. FcR γ chain deletion results in pleiotrophic effector cell defects. (1994). Cell 76:519-529.

Takatsu,K., T.Hamaoka and M.Kitagawa. Antibody production in mice VI : effect of anti-carrier antibody on cellular co-operation in the primary anti-hapten antibody response. (1974). Immunology 26:233-246.

Tony,H.P and A.Schimpl. Stimulation of murine B cells with anti-IgG antibodies : dominance of a negative signal mediated by the Fc Receptor. (1980). Eur. J. immunol. 10:726-729.

Uh,J.W and J.B.Bagman. The suppression of antibody formation by passively administered antibody. (1960). J. Exp. Med. 113:935-957.

Van Reagan,N. Direct intrafollicular differentiation of memory B cells into plasma cells. (1990). Immunology Today 11(5):154-157.

Wason,W.M., Regulation of the immune response with antigen specific IgM antibody : a dual role. (1973). J. Immunol. 110(5):1245-1252.

Wiersma,E.J., P.G.Coulie and B.Heyman. Dual immunoregulatory effects of monoclonal IgG-antibodies : suppression and enhancement of the antibody response. (1989). Scand. J. Immunol. 29:439-448.

Wiersma,E.J. Enhancement of the antibody response to protein antigens by specific IgG under different experimental conditions. (1992). Scand. J. Immunol. 36:193-200.

Wiersma,E.J., M.Nose and B.Heyman. Evidence of IgG-mediated enhancement of the antibody response *in vivo* without complement activation via the classical pathway. (1990). Eur. J. Immunol. 20:2585-2589.113

Yang,S.Y., E.Milford, U.Hammerling and B.Dupont. Description of the reference panle of B-lymphoblastoid cell lines for factors of the HLA system: the B-cell line panel designed for the tenth international histocompatibility workshop. In: Dopont,B., ed., Immunology of HLA : vol.1 histocompatibility testing 1987. New York: Springer-Verlag; 1987:11-19.