APPLICATION OF IMMUNOMAGNETISM FOR ANTIGEN PREPARATION AND CELL SELECTION IN THE CREATION OF HYBRIDOMAS

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Application of Immunomagnetism for Antigen Preparation and Cell Selection in the Creation of Hybridomas

by

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ABSTRACT

As part of a larger program to make monoclonal antibodies to HLA-DP polymorphisms, the possibility of using immunomagnetism to replace some cumbersome procedures in hybridoma creation was investigated. In the first part of this study, metallic beads were coated with anti-DP capture antibody and then were used for immune absorption of DP molecules from cell lysates. The absorbed molecules were identified with the help of a fluorescent anti-DP antibody and flow cytometry. Optimum conditions for absorption of DP molecules onto beads were worked out. In the second part, the immunogenic properties of the purified DP molecules were tested, in which metallic beads coupled with absorbed molecules were injected into mice to provoke an immune response. Serum antibody corresponding to three different antigen doses was determined and showed that the strong antibody response was correlated with the large dose of antigen. The influence of beryllium sulphate and complete Freund's adjuvant on these antibody responses was compared. In the final part of the study, immune spleen cells were sorted by using goat anti-mouse IgG coated metallic beads and/or antigen coupled beads. Following magnetic separation the selected spleen cells were fused with SP2/o myeloma cells

without the detachment of the beads, using a centrifugal-electrofusion technique. Hybridomas which secreted antibodies were examined for their specificity to DP polymorphisms. The frequency of positive hybridomas, in fusions of pre-selected cells, was compared with that of the fusion of unsorted cells (prior to selection) and with that of the remainder of the cells left behind after a sort. Once a variety of tissue culture problems had been resolved, there was preliminary evidence suggesting that pre-fusion selection of antigen-specific cells can be a helpful and time-saving procedure.

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Chapter 1

INTRODUCTION

The success of transplantation of organs or tissues depends largely upon having the graft donor and the recipient as closely matched for tissue antigens as possible. In the case of blood transfusion this is a familiar situation, where the antigens on the red blood cells in the transfused blood need to be matched with those of the recipient or, at the very least, compatible with the recipient. In the case of other tissues such as bone marrow, or of organs, the matching involves particularly a series of antigens that are found on most of the nucleated cells of the body. For convenience these antigens are typed using white blood cells, so they are also known as human leucocyte antigens (HLA). The system of antigenic molecules which has the major effect on transplantation success is coded by genes on the short arm of chromosome 6 in the human. These genes are known as the major histocompatibility complex (MHC), and they are divided into MHC class I, comprising HLA-A, -B, -C and MHC class II, comprising HLA-DR, -DQ and -DP. All the class II products are composed of two polypeptide chains, and both of them are

polymorphic.

The HLA-DP system was the most recently discovered and was revealed in 1980 by use of the primed lymphocyte test (PLT) (Shaw et al. 1980). This test depends upon culturing white blood cells for several days and it is a cumbersome, specialized technique that was not easily adapted for use in a routine tissue typing laboratory; it remained in the research domain. However, it seemed likely that these polymorphic molecules were important determinants of transplantation outcome and so a serological typing method was highly desirable.

Our laboratory in Memorial University of Newfoundland decided to attempt to make monoclonal antibodies to the polymorphism of HLA-DP using a mouse system. In the 1980's the fact that HLA-DP can be recognized serologically was shown by the work of Heyes et al. (1986) who made a mouse monoclonal antibody to HLA-DP and by Johnson (1986) who raised an allo-antiserum in a human volunteer by deliberate immunization. Since those promising beginnings there has not been much progress, despite the efforts of many laboratories. It turns out that the production of antibodies to HLA, especially to HLA-DP is a difficult undertaking.

There are several problems involved in making monoclonal antibodies against DP. First, it is not easy to obtain purified HLA-DP molecules as immunogen. As was

suggested in the paper of Eckels et al. (1986) and the paper of Johnson et al. (1986), the expression of DP molecules on the cell membrane is a relatively low percentage of the total amount of class II HLA molecules expressed and is not stable. That is, a large number of cells is required. Moreover, the conventional method for purification of membrane molecules is painstaking and time-consuming. The application of gene transfection for this type of work has been a significant advance. However, the use of DP transfectants for producing hybridomas specific for HLA-DP appears to be less successful than was originally anticipated. Along with the reduced expression of DP molecules on cells, it seems that such molecules may be less immunogenic, for the mouse, than are other molecules, for example, HLA-DQ. This means that the frequency of specific hybridomas is low. Thus many thousands of hybridoma cultures must be screened in order to find any positives. In many experiments there may be none.

To overcome these problems an alternative approach was suggested, using the technique of immunomagnetism. In this technique, metallic beads can be coupled to anti-DP antibodies and can serve as a solid phase for affinity purification of DP molecules. There were three attractive features to this technique, 1) the beads are easily manipulated with a magnet; 2) the beads are small enough and non-toxic so that, when loaded with DP molecules, it seemed likely that they could be

injected into mice as immunogen. In this regard the work of Nillson et al. (1990) is relevant since they showed that antigens immobilized on a carrier (like Sepharose beads, nitrocellulose membrane etc.) could be injected intrasplenically and induce an antibody response with a reduced amount of antigen, in the nanogram range; 3) the potential existed to use these DP coated beads as a reagent for cell sorting, using immunomagnetism, to extract B cells whose surface immunoglobulin is specific for DP epitopes. Such B cells could in principle, be used for fusions with the expectation that this would result in much higher frequency of positive hybridomas. For these three reasons this technique was chosen as the subject for investigation in this study.

Chapter 2

LITERATURE REVIEW ON IMMUNOMAGNETISM

A. HISTORY OF DEVELOPMENT OF IMMUNOMAGNETISM

During the last decade, immunology has seen the introduction of an immunochemical technology in which magnetic fields play an important part; thus molecules, or cells in suspension can be manipulated by the application of magnetic fields following exposure to metallic beads. This recent phase was heralded by earlier work in which iron particles were used.

The early use of magnetism in immunology dates from 1934, in which Rous et al. first isolated hepatic reticuloendothelial cells (Kupffer cells) by using magnetic fields. In their experiment, Rous et al. (1934) injected an animal with carbonyl iron particles, causing Kupffer cells to take them up. They then killed the animal and perfused the liver, collecting the iron-containing cells in the perfusate by means of an electromagnet. This experiment was the beginning of the exploration of the use of magnetic fields in cell separation. Prompted by Rous' work, Heidelberger et al. (1946) studied the use of the magnetic property of "haematin

or related malarial pigments" in malaria infected red blood cells for concentration of malaria parasites. They took blood samples from patients with malaria and exposed them to a magnetic field, thus separating the parasite infected red blood cells from the normal ones with about 15 fold increase in concentration. A few years later, Levine (1955) fed the phagocytes in a blood sample carbonyl iron particles in vitro, then separated the phagocytes from other peripheral white blood cells using an electromagnet. Levine's method became a classical procedure to remove phagocytes from cell suspensions (Hudson et al. 1976).

A remarkable development was made in the 1970's, which was based on the observation that the removal of reversibly bound oxygen from haemoglobin in red blood cells results in a change of the iron-haem from a non-magnetic to a paramagnetic state. Melville et al. (1975) treated human red blood cells with sodium dithionite to reduce oxyhaemoglobin to deoxyhaemoglobin, then separated the red blood cells from the whole blood by applying a strong magnetic field. Owen et al. (1978, 1979) extended this experiment. First, they changed the sodium dithionite to sodium nitrite which is less damaging to red blood cells; then, they took nitrite-treated sheep red blood cells to rosette human T lymphocytes and then removed all the red cells as well as the rosetted T cells from the suspension using an electromagnet. Owen et al. (1982) also

extended these experiments to allow specific separation of antibody secreting cells. They used amino acid polymers as antigen to immunize mice. Then they coupled the antigen to nitrite-treated sheep red blood cells so that the red cells would bind to antigen-specific lymphocytes in a mouse spleen cell suspension. The rosetted lymphocytes were then separated from the rest of the spleen cells using a magnet. This experiment of Owen and his colleagues was an important step towards the development of targeting magnetic material to specific cell types.

Later development of the magnetic separation technique involved the preparation of magnetic solid-phase materials to which enzymes or antibodies could be coupled. The advantage of this was that the magnetic (metallic) particles carrying the enzymes or antibodies could be easily manipulated, particularly for washing away unbound reagents. The earliest report of this application appears to be that of Robinson et al. (1973). They made iron oxide-cellulose particles for coupling to α-chymotrypsin and β-galactosidase. Those particles were magnetised because they were made with magnetic iron oxide. This property meant that they tended to form aggregates at low stirring speeds in a reactor and could be removed as sediment. For full enzyme activity, deaggregation was easily brought about by higher stirring speeds. Thus enzymes could be added to, or removed from, reaction mixtures

of various types. Shortly thereafter, Hersh et al. (1975) made silane-coated iron oxide particles for immobilizing digoxin antiserum. Using these antibody coupled metallic (and also magnetic) particles, they did a radioimmunoassay. They used a magnet to remove the antibody coated particles and in this way were able to separate bound from unbound antigen. Lynn Nye et al. (1976) repeated the last experiment but with iron oxide particles coated with m-diaminobenzene polymers. Metallic Sepharose beads (Mosbach et al. 1977) and iron-oxide treated starch particles (Mosbach et al. 1979) were also prepared for affinity chromatography and for targeting of drugs, respectively.

There were two further advances in the development of magnetic solid phase techniques. One was made in 1977 by Guesdon et al. who prepared metallic polyacrylamide beads for use in an enzyme immunoassay. In their preparation, Guesdon et al. introduced active aldehyde groups (-CHO) into the beads, to which antibodies or other proteins could be directly coupled in a single step, thereby eliminating cumbersome intermediate procedures. Another advance was made by Molday et al. (1977) who first applied a magnetic solid phase technique to antibody-based cell isolation. Molday et al. coupled metallic particles with goat anti-mouse Ig antibodies, thus targeting the particles to mouse B lymphocytes which express IgG or IgM molecules on their surface membranes. When mouse

spleen cells were incubated with those particles, the Ig expressing cells became rosetted with the metallic particles, thereafter, particles and B cells could be separated from the other cells by the application of a magnetic field. The significance of this experiment is that due to the high serological specificity of the antibodies coated onto the metallic particles, immunomagnetic separation became a potent method for specific separation of defined cell types from a complex mixture of cells.

Immunomagnetic separation became a widely used method in immunology after the development of a method for preparing metallic beads of uniform size. Ugelstad et al. (1983) developed such a procedure for making metallic beads from styrene divinylbenzene. Using this method, the metallic beads were nearly identical in size, density and iron oxide content. Each bead comprised an iron core and a polymer shell. Functional groups could thus be incorporated into the surface of the beads, which could be modified for physical absorption or covalent coupling of antibodies or other proteins. The newly developed metallic beads quite soon became commercially available, which allowed their widespread use.

B. THE APPLICATION OF METALLIC BEADS

B.1. Removal of tumour cells from bone marrow

A large dose of chemotherapy and/or radiotherapy is often required for treatment of malignant diseases. Compared with other tissues, bone marrow is more vulnerable to drug and radiation therapy. One option to overcome this problem is to collect a portion of bone marrow before high dose cytotoxic therapy is given. It is then reinfused to rescue the haemopoietic system after the treatment (autologous bone marrow transplantation). However, in cases with metastatic tumours, malignant cells would be harvested together with the normal bone marrow cells. Reinfusion of such cell suspensions could lead to reseeding of tumour cells along with the normal progenitor cells. Metallic beads have provided a useful tool for depleting tumour cells from such bone marrow samples. Poynton et al. (1983) first reported the application of immunomagnetic separation to remove leukaemic cells from a patient's bone marrow in the treatment of a child with common acute lymphocytic leukaemia (cALL). They harvested bone marrow from the child with cALL and incubated the bone marrow cells with metallic beads coated with monoclonal antibodies against the cALLA surface antigen (now CD10) of leukaemic cells. The

tumour cells were then separated from the normal ones using a magnetic field. After the high dose cytotoxic drug therapy, the tumour cell depleted bone marrow cells were reinfused to restore the normal bone marrow function. The marrow was evidently repopulated quite soon, but long term outcome was not reported.

Metallic beads were used by Treleaven et al. (1984) for removal of neuroblastoma cells from bone marrow in the treatment of patients with metastatic neuroblastoma. In order to maximize tumour-cell depletion, a panel of six different mouse IgG anti-neuroblastoma antibodies was used first to sensitize target cells, which, thereafter, were removed by adding metallic beads coated with anti-mouse Ig, followed by magnetic separation. Again the re-engraftment after chemotherapy was successful, but long term outcome was not reported.

Numerous similar approaches have since been described. In recent years, immunomagnetic purging of breast cancer cells and lung carcinoma cells from the bone marrow of the patients with advanced malignant diseases has been studied (Anderson et al. 1989; Vredenburgh et al. 1990, 1991). Although no randomized clinical trial has so far been conducted comparing autologous bone marrow transplantation with and without *in vitro* marrow purging, the use of this procedure has been regarded by some laboratories as a routine part of the

treatment for patients undergoing autologous bone marrow transplantation (Pole et al. 1990).

B.2. Depletion of T lymphocytes from bone marrow

Allogeneic bone marrow transplantation has been the treatment of choice for aplastic anemia, leukaemia and other disorders of haematopoietic system (O'Reilly 1983). One of the major obstacles to the success of allogeneic bone marrow transplantation is graft versus host disease (GVHD) which is initiated by immunocompetent donor T lymphocytes responding to alloantigens expressed on host cells. In attempts to avoid this complication by depletion of T lymphocytes from the marrow inoculum, the immunomagnetic separation method was examined. In 1987, Vartdal et al. reported using metallic beads coated with monoclonal antibodies specific for T cell CD2 and CD3 antigens to remove T lymphocytes from human bone marrow cell suspensions. Shortly after that, several similar reports were published (Geisler et al. 1989; Frame et al. 1989; Gee et al. 1989). However, it is not yet clear if immunomagnetism has real advantages for purging T cells, when compared with other methods, such as antibody plus complement or antibody coupled to a cytotoxic molecule.

B.3. Positive and negative cell selection for functional assays

There have been many reports about the use of metallic beads for cell selection. The use of metallic beads for positive cell selection can be illustrated by the earlier work of Gaudernack et al. (1986) and Sollid et al. (1986) who isolated $CD8^+$ T lymphocytes from peripheral blood by rosetting cells with metallic beads coated with antibody against CD8 membrane molecules. With these purified cells they studied the functional properties of $CD8^+$ T cells in response to allogeneic cells and soluble antigens. In their experiments, the presence of metallic beads appeared not to interfere with the effector function of the selected T lymphocytes.

Another example of the application of metallic beads to positive cell selection is the study of lymphocytes isolated from synovial fluid of patients with arthritis (Hovdenes et al. 1989a, 1989b and 1989c). In this study, metallic beads were coated with antibodies against CD4 or CD8 molecules and were mixed with synovial fluids from patients with rheumatoid arthritis and other types of arthritis. $CD4^+$ or $CD8^+$ T lymphocytes were isolated from the synovial fluid as they rosetted with the beads and were compared with the normal T lymphocytes from healthy peripheral blood with respect to their ability to proliferate in response to mitogens, produce interleukin 2 and gamma interferon and to express activation

markers.

It has been found that metallic beads bound to cells during selection can be detached by incubating rosettes at 37° C for 16-20 hours (Lea et al. 1986; Funderud et al. 1990). However, the detachment of beads only occurred in certain cells. Nilsson et al. (1987) has reported in a study with Langerhans' cells that metallic beads could not be detached from cells even if the rosettes were cultured for 24 hours and pipetted vigorously. It was assumed that the easy detachment of beads from B cells is due to relatively frequent antigen shedding on B cell membranes (Funderud et al. 1990). Recently, Rasmussen et al. (1992) have described a new method for detachment of metallic beads from positively selected B lymphocytes. In this method, anti-Fab antibody was used to separate the bond between the anti-mouse Ig antibody on metallic beads and the surface Ig of B lymphocytes; whether this is due to the competitive binding mechanism or the result of a conformation change, which affects the antigen binding site, cannot be stated at present.

An example of the application of negative cell selection using metallic beads is a study on the role of antigen presenting cells (Tjernlund et al. 1988). In this study, Tjernlund and his colleagues removed Langerhans' cells from epidermal cell suspensions using metallic beads coupled with anti-CD1 antibodies. Then they examined T-cell response

to purified protein derivative (PPD) in these suspensions. This experiment gave strong evidence that keratinocytes do not take part in antigen presentation even though they are found to be HLA-DR-expressing under certain conditions.

B.4. Tissue typing

The application of immunomagnetic techniques to serologic tissue typing has been described in several reports. These reports describe two ways to make use of metallic beads for phenotyping lymphocytes. In the first, the cells to be typed are isolated from peripheral blood by positive cell selection using metallic beads coupled with antibody specific for a defined cell type. Then, without removal of the beads, they are made to react with typing sera and complement. The results are read in a fluorescence microscope employing a mixture of ethidium bromide and acridine orange to monitor the viability of the cells (Vartdal et al. 1986, 1987; Povlsen et al. 1988). This method was compared with the conventional Kissmeyer-Nielsen technique (Vartdal et al. 1986; Povlsen et al. 1988, 1991). It was shown that the phenotyping assay with the use of immunomagnetic technique had advantages over the conventional one. First, the donor's cells could be obtained quickly with good purity and viability. This time-saving

procedure was preferred for phenotyping cadaveric donors as it helps to prevent a prolonged cold ischemia time. Second, as purified cells of the specific type were used in the assay, the sensitivity was increased, particularly in HLA class II typing.

Another way of using metallic beads for tissue typing is based on identification of cells that have formed rosettes with metallic beads conjugated with monoclonal antibodies directed against cell-surface antigens. In a study by Hansen et al. (1987), monoclonal antibodies specific for HLA-B27 were coupled to metallic beads. When these beads were mixed with a small amount of whole blood, they formed rosettes with HLA-B27-bearing leucocytes. Removal of red cells and other debris by washing in either lysis buffer or PBS was achieved with the aid of a magnet, to immobilize the beads while the supernatant fluid was "flicked" off. This method allowed typing for HLA-B27 to be accomplished in about 20 min by microscopic evaluation of rosette formation. In another study of Skjonsberg et al. (1990), monoclonal antibodies against antigens on myeloma or lymphoid cells were coupled to metallic beads. Phenotyping of acute leukaemias was then performed. Comparison of the rosette assay with conventional microcytotoxicity assays showed in both studies that with the use of metallic beads the sensitivity was increased.

B.5. Antigen-specific cell selection

An interesting use of immunomagnetic separation in immunology is the enrichment of specific antibody secreting cells using antigen-coupled metallic beads. There are two examples of such an application. One is the experiment of Egeland et al. (1988) who coupled metallic beads with rabbit IgG and used these beads to rosette rheumatoid factor positive B lymphocytes from peripheral blood of patients with rheumatoid arthritis. This method is based on the fact that the Fc fragment of rabbit IgG is specifically recognized by rheumatoid factors of IgG, IgM and IgA isotypes. The rosetted RF secreting B cells were collected using a magnet and were transformed with Epstein-Barr virus for bulk expansion. Unfractionated B cells from RA patients were also transformed as controls. The frequency comparison in this experiment showed that 10^3- to 10^4-fold enrichment of antigen-specific B lymphocytes was obtained using these antigen coupled beads.

Another example of such application is the work of Ossendorp et al. (1989), in which antigen coupled metallic beads were used for selection of high affinity B cell hybridomas. In an attempt to obtain high affinity thyroglobulin-specific antibody secreting hybridomas, Ossendorp and his colleagues coupled thyroglobulin to metallic beads and incubated them with bulk cultures of hybridoma cells, obtained by fusing immune spleen cells from

thyroglobulin immunized mice with myeloma fusion partner cells. The rosetted hybridoma cells were isolated from the unrosetted cells and subsequently cultured and tested. It was shown in their experiment that a 300-fold enrichment of thyroglobulin-specific hybrid cells was obtained using this method. These two experiments provided a possible circumvention of the problem associated with low frequency of specific antibody secreting hybrids in attempts to create specific hybridomas.

This literature search did not find any reports of the use of immunomagnetism for biochemical purification of molecules, nor for the pre-fusion selection of antigen-specific B cells for making hybridomas. Both of these were explored in this thesis.

Chapter 3

OBJECTIVES

The objective of this thesis was to explore the possibility of using techniques of immunomagnetism to replace certain cumbersome procedures in monoclonal antibody production, specifically (1) a simple method was sought to purify HLA-DP molecules from cell lysate and (2) it was hoped to improve the yield of fusions using HLA-DP immunized mice, by pre-fusion enrichment for putative antigen-specific cells. This overall objective can be broken down into 3 parts.

1. To couple anti-DP antibody to metallic beads and to use them to extract HLA-DP molecules from cell lysates.
2. To evoke antibody responses in mice by immunizing them with metallic beads absorbed with HLA-DP molecules.
3. To enrich IgG-bearing or antigen-specific B lymphocytes from mouse spleen cell suspensions using anti-IgG or antigen coupled metallic beads, respectively, and to fuse the selected cells with myeloma cells by application of the centrifugal electrofusion technique.

Chapter 4

MATERIALS AND METHODS

A. CAPTURE OF DP MOLECULES ONTO METALLIC BEADS

A.1. Determination of the optimal concentration of anti-DP antibodies for binding transfectant cells

Materials

NFLD.M58: A monoclonal antibody specific for an HLA-DP polymorphism which includes DPB1*0201, DPB1*0301, DPB1*0402, DPB1*0901 and DPB1*1001 (Drover et al. 1991).

NFLD.M67: A monoclonal antibody specific for a monomorphic determinant on HLA-DP (Marshall et al. unpublished).

(Both antibodies are used as hybridoma culture supernatants from the Immunology laboratory of Memorial University of Newfoundland)

Secondary antibody: Affinity purified $F(ab')_2$ fragments of goat anti-mouse IgG + IgM, conjugated with fluorescein isothiocyanate (FITC) (Jackson Immunoresearch laboratories, West Grove, PA).

Culture and washing medium: Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, $5x10^{-5}$M 2-mercaptoethanol, 500units/ml

penicillin, 500μg/ml streptomycin and 0.25mg/ml geneticin (G418 sulphate) (DMEM, FCS, L-glutamine, penicillin and streptomycin were all from GIBCO Laboratories, Grand Island; 2-mercaptoethanol was from Sigma Chemical Co., St Louis, MO). L25.4 transfectant cells: These are HLA-DP-expressing L-cell transfectant cells containing DPA1*0101 and DPB1*0402 genes (Lair et al. 1988; Klohe et al. 1988). The cells were grown in culture medium at 37°C in 10% CO_2 atmosphere at a density of $2x10^6$/dish, and harvested 48 hours after being treated with trypsin.

Method

1). Monoclonal antibodies M58 and/or M67 in concentrated, untreated or serially diluted solutions were prepared.

2). $2x10^5$ L25.4 transfectant cells were prepared in each of ten tubes.

3). The cells were washed twice with ice cold washing medium by spinning at 200xg for 5min.

4). The cells were incubated with appropriate dilutions of M58 or M67 (100μl) at 4°C for 30min.

5). The cells were washed three times with ice cold washing medium. All supernatant was removed.

6). The cells were incubated with the secondary antibody (100μl) at 4°C for 30min.

7). The cells were washed three times as before.

8). The cells were resuspended in 1ml of washing medium;

they were then ready for flow cytometric analysis.

A.2. Cross Blocking of I3 by M58 or M67

Materials

I3 is a monoclonal antibody specific for a monomorphic determinant on MHC class II molecules; it is conjugated with FITC (Coulter Immunology, Hialeah, FL). Flow cytometric analysis of the antigen specificity of I3 using transfectant cells as targets showed the following results:

	L25.4 (DPB1*0402)	DP8303 (DPB1*0401)	DP8306 (DPB1*0901)	DQ8205 (DQB1*0201)	DR8131 (DRB1*1001)	DR8137 (DRB4*0101)	DR8114 (DRB1*04..)
I3	+	+	+	-	-	-	±

This data suggests but does not prove that it is largely specific for HLA-DP with very little binding to DQ or DR molecules.

NFLD.M58: See above

NFLD.M67: See above

L25.4 transfectant cells: See above

Washing medium: See above

Method

L25.4 transfectant cells ($2x10^5$) were washed twice with ice cold washing medium at 200xg for 5min. They were then incubated with M58 or M67 (100μl) at 4°C for one hour. The

cells were washed three times again and incubated with I3 (10μl) at 4°C for half an hour. After three washes to remove free I3, The cells were resuspended in 1ml of washing medium and analyzed by flow cytometre.

A.3. Coating metallic beads with anti-DP antibody

Materials

Metallic beads: Covalently coupled with goat anti-mouse IgG (Dynal Inc., Great Neck, NY)

Anti-DP antibody: M67 and/or M58 (see above)

Washing solution: Phosphate buffered saline, pH7.4, containing 1% fetal calf serum (PBS/FCS).

Magnetic particle concentrator (Dynal Inc., Great Neck, NY)

Method

The metallic beads were suspended by lightly shaking the bottle. The desired amount of beads ($4x10^8$/ml) were drawn into a syringe and transferred to a 15ml centrifuge tube. The beads were washed twice with PBS/FCS by attaching the tube to a magnetic particle concentrator for one minute, followed by pouring off the supernatant. After washing, the metallic beads were resuspended in M67 or M58 monoclonal antibody solution and then were incubated on a rotator at 4°C overnight. The following day, the metallic beads with M58 or M67 antibodies

attached were washed four times as before so that the free antibody molecules were removed.

A.4. Preparation of cell lysates

Materials

Human B cell lines: HO3O1(DPB1*0501), COX(DPB1*0301), SAVC(DPB1*1001) and WT51(DPB1*0201) (S. Y. Yang et al. 1989)

Transfectant cells: DP8303 and DP8306 bearing DPB1*0401/DPA1*0101 and DPB1*0901/DPA1*0201 respectively (Lair et al. 1988; Klohe et al. 1988)

B cell line culture medium: RPMI-1640 supplemented with 10% fetal calf serum, 2mM L-glutamine, 1% sodium pyruvate, 500units/ml penicillin and 500μg/ml streptomycin

Transfectant cell culture medium: DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine, $5x10^{-5}$M 2-mercaptoethanol, 500units/ml penicillin, 500μg/ml streptomycin and 0.25mg/ml geneticin (G418 sulphate) (for DP8306 cells) or 1% hypoxanthine/methotrexate/thymidine (HMT) (for DP8303 cells)

Lysing buffer: 0.15M NaCl, 0.01M Tri-HCl, pH8.2, 1mM EDTA, 2mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA)

Note: RPMI, DMEM, L-glutamine, penicillin, streptomycin and fetal calf serum were all from GIBCO, Grand island, NY; 2-

mercaptoethanol was from Sigma Chemical Co., St. Louis, MO.

Method

Cell lysates were prepared as previously described by Schneider et al. (1982) and Goding (1986). Human B cell lines were grown in the B cell line culture medium at 37°C in 5% CO_2 atmosphere at a density of $2x10^5$/ml; transfectant cells were grown in transfectant cell culture medium at 37°C in 10% CO_2 atmosphere at a density of $5x10^6$/dish. To prepare cell lysates, the cells were washed twice with ice cold cell culture medium at 200xg for 5min, and then resuspended in ice cold lysing buffer. For each $1x10^8$ cells , 1-2ml lysing buffer was added. After incubating at 4°C for 20min, the lysate was centrifuged for 30min at 3000xg, 4°C to sediment nuclei and debris. The supernatant was decanted and ready for use.

A.5. Absorption of DP molecules from cell lysates

After being coated with anti-DP antibody, metallic beads developed affinity for DP molecules and acted as a solid phase for immune absorption of DP molecules. The absorption was performed by mixing the metallic beads with the cell lysate and incubating the mixture on a rotator at 4°C overnight. The following day, the metallic beads were collected and washed once with ice cold lysing buffer and

twice with cold PBS/FCS, using a magnetic particle concentrator to retain the beads while the supernatant fluid was poured off and discarded. After the last wash the beads were resuspended in PBS/FCS.

A.6. Staining DP-absorbed beads with I3 antibody

After the above absorption the metallic beads were first incubated with normal mouse serum at 4°C for 30min to block nonspecific binding sites. Then, the mouse serum was removed using a magnet to retain the beads, and the beads were incubated with 10μl of I3 for another 30min at 4°C. After that, they were washed three times with ice cold PBS/FCS and resuspended in 1ml of the same buffer for flow cytometric analysis.

A.7. Specific blocking of I3 by M58 binding to DP-absorbed beads

Materials

I3: See above

NFLD.M58: A monoclonal antibody specific for a DP polymorphism (see above)

Metallic beads with absorbed molecules derived from a cell

lysate made from WT51 B cell line cells (DPB1*0201/DPA1*0101).

Washing buffer: PBS/FCS (see above)

Method

After absorption of DP molecules from a cell lysate and blocking nonspecific staining using normal mouse serum (see Method above), one million metallic beads were incubated with M58 (200µl) at 4°C for 30min with intermittent stirring. After that, the M58 supernatant were removed using a magnet, and the fluorescent antibody I3 was added. Another incubation was then done under the same conditions. This was followed by three washings with PBS/FCS using a magnet and the beads were resuspended in 1ml of the same buffer for flow cytometric analysis.

A.3. Staining DP-absorbed beads with biotinylated M67

Materials

Biotin-M67: Monoclonal antibody against a DP monomorphism (see above) conjugated with biotin, 1/10 diluted (Immunology laboratory, Memorial University of Newfoundland).

Avidin-FITC: 1/100 dilution (Calbiochem, La Jolla, CA 92037).

M58 coated onto metallic beads: See above

Cell lysate: Made from WT51 B cell line cells (see above)

Method

Immune absorption of DP molecules from a cell lysate using M58 coated metallic beads was performed as described above. Then, the beads were blocked with normal mouse serum by incubating them at 4°C for 30min. After that, 100μl biotin-M67 was added to the beads and another incubation was done under the same conditions. After three washings with PBS/FBS, the metallic beads were stained with 100μl avidin-FITC by incubating them at 4°C, 30min. Finally, the metallic beads were washed three times with PBS/FBS and resuspended in 1ml of the same solution for flow cytometric analysis.

B. IMMUNIZATION METHODS

B.1. Primary immunization

Materials

Immunogen: DP-absorbed metallic beads in sterile PBS

Adjuvant: Complete Freund's adjuvant

Mice: Balb/c and C3H mice aged from 2-6 months.

Method

One volume of metallic beads was emulsified with two volumes of complete Freund's adjuvant using two syringes connected via a double ended fitting. 300μl of the emulsion

was then injected into each mouse subcutaneously at 2 or 3 sites on the back.

B.2. Secondary immunization

Materials

Immunogen: DP-absorbed metallic beads suspended in sterile PBS

Adjuvant: Incomplete Freund's adjuvant

Mice: Same as in the primary immunization

Method

The secondary immunization was done four weeks after the primary immunization. One volume of metallic beads was emulsified with two volumes of incomplete Freund's adjuvant. 300μl of the emulsion was then injected into each mouse intraperitoneally.

B.3. Antigen boost before splenectomy and fusion

Materials

Immunogen: DP-absorbed metallic beads; or the cells used for preparation of cell lysates.

Sterile PBS

Method

The boost was done two days before fusion. $5x10^7$ beads absorbed with DP molecules were suspended in 100μl of PBS, then they were injected intrasplenically; or $2x10^7$ cells were suspended in 100μl of PBS, then they were injected intravenously via the tail vein.

B.4. Collection of immune mouse serum

The mouse were lightly warmed in a procedure room by raising the room temperature to about 26-28°C. The last 2-3mm of the tail was then chopped off with a clean scalpel blade. Several drops of blood were collected in this way into a microcentrifuge tube which is left at 4°C overnight to allow the clot to retract. The following day, the serum was recovered after centrifugation to sediment the red cells and clot. The serum was transferred to an Eppendorf tube and was stored frozen until ready for use.

C. ANTIBODY MEASUREMENT BY CELISA

C.1. Assay of specific antibody using B cell line cells as antigen

Materials

Antigen (Target): Human B cell line cells HO301, COX, SAVC and WT51 (these were the ones used for immunizations).

First antibody: Mouse serum or hybridoma supernatant under test

Second antibody: Affinity purified $F(ab')_2$ fragments of goat anti-mouse IgG and IgM conjugated with horse radish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA), 1/2500 in PBS containing 2% bovine serum albumin.

Washing buffer: Phosphate buffered saline containing 0.5% bovine serum albumin (Sigma Chemical Co, St Louis, MO)

Poly-L-Lysine (PLL) (1mg/100ml): 10mg PLL (Hydrobromide, molecular weight 180,000, from Sigma Chemical Co.) in 1000ml PBS, stored at 4°C.

Phosphate citrate buffer, pH5.0:

A. 0.1M citric acid solution: 1.92g citric acid in 100ml distilled water

B. 0.2M phosphate solution: 2.82g phosphate in 100ml distilled water

Mix 4.9ml citric acid solution with 5.1ml phosphate solution

and 10ml of distilled water.

Substrate: 4mg of orthophenylenediamine (OPD) dissolved in 10ml phosphate citrate buffer. When OPD is completely dissolved, 4μl of 30% H_2O_2 is added.

2.5N H_2SO_4

Plates: 96-well U-bottom polyvinyl chloride microtitration plates (PVC) and 96-well Enzyme-Immunoassay microplates

Method

1). Target cells were washed one time with washing buffer at 200xg for 10min and plated on PVC plates at $5x10^4$/10μl/well.

2). 25μl of diluted mouse serum or hybridoma supernatant was added to each well and incubate with the cells at room temperature for one hour.

3). The cells were washed 3 times by adding 125μl washing buffer to each well, spinning at 150xg for 5min at 8°C and flicking off supernatant.

4). The second antibody was added with 50μl per well and incubated at room temperature for one hour.

5). In the mean time, EIA plates were coated with 75μl PLL per well. After 30min, the plates were drained by flicking off supernatant.

6). The cells were washed four times as before.

7). The U-bottom PVC plates were fitted onto the EIA plates. 100μl washing buffer was added to each well, a hole was

punched in the centre of each well using an 18 gauge needle.

8). The plates were spun at 150xg for 7min, then the PVC plates were removed and the supernatant was flicked off.

9). Substrate was added, 100μl/well; the plates were left in the dark for 30min.

10). 50μl H_2SO_4 was added to each well to stop the colour development.

11). The plates were read in Multiscan Spectrophotometer at 490nm wavelength.

C.2. Assay of specific antibody using transfectant cells as targets

Materials

Target cells: Transfectant cells DP8303 and DP8306 (see above)

First antibody: mouse serum or hybridoma supernatants under test

Second antibody: Affinity purified F(ab')2 fragments of goat anti-mouse IgG and IgM conjugated with horse-radish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA), 1/2500 diluted in PBS containing 2% bovine serum albumin.

Washing buffer: Phosphate buffered saline containing 0.05%

Tween-20 detergent

Phosphate citrate buffer, pH5.0: See "Assay using B cell line cells as target".

Substrate: See "Assay using B cell line cells as target".

2.5N H_2SO_4

Plate: 96-well tissue culture plate

Method

1). Transfectant cells were plated in tissue culture plates, $5x10^4$ cells in 100μl DMEM culture medium(see above) per well; and incubated at 37°C overnight.

2). The cells were washed one time by adding 150μl washing buffer to each well and then flicking it off.

3). 25μl diluted mouse serum or hybridoma supernatant was added to each well, and incubated at room temperature for one hour.

4). The cells were washed four times as before.

5). The second antibody, 50μl per well, was added and incubated at room temperature for one hour.

6). The cells were washed four times as before.

7). Substrate, 100μl per well, was added and the plates were left in the dark for 30min.

8). 50μl H_2SO_4 was added to each well to stop the colour development.

9). Optical density was measured in Multiscan Spectrophotometer at 490nm wavelength.

D. CELL SELECTION AND HYBRIDOMA METHODS

D.1. Growing myeloma cells

Materials

Cell culture medium: DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine, 500units/ml penicillin, 500μg/ml streptomycin, $5x10^{-5}$M 2-mercaptoethanol (DMEM, FCS, L-glutamine, penicillin and streptomycin were all from GIBCO, Grand Island, NY; 2-mercaptoethanol was from Sigma Chemical Co., St Louis, MO)

Tissue culture flask

Method

SP2/o myeloma cells were grown in culture medium at 37°C in a 5% CO_2 atmosphere. Before fusion, the cells were kept growing at a density of $5x10^4$ to $1x10^5$ cells per ml. with a viability of 95%, for two to six weeks.

D.2. Obtaining the mouse spleen cells

The immunized mouse was killed with Ethrane followed by cervical dislocation, and was then sterilized by immersion in 70% ethanol. The mouse abdominal skin was incised

longitudinally and separated from the abdominal wall. Then, the abdominal cavity was opened and the spleen was dissected and removed to a petri dish containing 5ml cold DMEM. After trimming the fat away, the spleen was transferred to another dish with 5ml cold medium. The spleen cells were then squeezed out of the organ after making a small cut at both poles, gently massaging the spleen with two bent syringe needles mounted on syringes. When most of the spleen cells were squeezed out in this way, they were transferred to a 15ml centrifuge tube on ice. The cells are pipetted several times and allowed to stand for a while to let large aggregates settle out. The cell suspension was then transferred to another centrifuge tube and washed three times with cold medium at 200xg for 5min. After the final wash the cells were counted.

D.3. Selection of IgG-bearing B cells

Materials

Metallic beads covalently coupled with goat anti-mouse IgG (all subclasses) (Dynal Inc., Great Neck, NY)

Magnetic particle concentrator (Dynal Inc., Great Neck, NY)

Spleen cell suspension from an immunized mouse.

Cell culture medium: DMEM supplemented with 10% fetal calf

serum, 2mM L-glutamine, 500units/ml penicillin, 500μg/ml streptomycin, $5x10^{-5}$M 2-mercaptoethanol (DMEM, FCS, L-glutamine, penicillin and streptomycin were all from GIBCO, Grand Island, NY; 2-mercaptoethanol was from Sigma Chemical Co., St Louis, MO)

PBS containing 1% FCS

Method

The metallic beads were washed three times with PBS/FCS to remove cytotoxic preservative. Then they were mixed with spleen cells in 5ml ice cold culture medium. The mixture was incubated on a rotator at 4°C for one hour. After that the metallic beads and the cells bound to them were separated by attaching the tube to a magnet; while the beads were held on the wall, the supernatant and free cells were poured off. The bead-selected cells were then resuspended in 10ml medium and counted.

D.4. Selection of antigen-specific B lymphocytes

Materials

Metallic beads that had absorbed DP molecules from a WT51 B cell line cell lysate (DPB1*0201)

Spleen cell suspension from a Balb/c mouse immunized with DP molecules from WT51 B cells.

Cell culture medium: See above

PBS containing 1% FCS

Method

DP absorbed metallic beads were washed three times with PBS/FCS, then mixed with spleen cells. The mixture was incubated on a rotator at 4°C for one hour. After that the metallic beads and the cells bound to beads were separated from free cells as described before. The selected cells were resuspended in 10ml cell culture medium and then counted.

D.5. Centrifugal-Electrofusion of bead-selected cells

Materials

Fusion buffer: It is a low ionic strength sucrose-phosphate buffer made up as follows: First, mix 21.6ml of 1/15M KH_2PO_4 solution with 54.2ml of 1/15M Na_2HPO_4 solution, adjust pH value to 7.2 and add distilled water to 500ml; then add 42.8g sucrose and 0.1017g $MgCl_2.6H_2O$ to the final solution.

Centrifugal electrofusion facility (Designed and constructed in Memorial University of Newfoundland): The electrical pulsing circuit has been described by McCormick et al. (1992). For these experiments it was connected to specially designed centrifuge tubes that have built-in electrodes. The apparatus was made in such a way that cells, sedimented into a pellet in

the centrifuge tube, could be exposed to brief high voltage pulses whilst the centrifuge was rotating.

Cell culture medium: DMEM supplemented with 20% FCS

Method

The bead-selected cells without detachment of beads were mixed with SP2/o myeloma cells in a ratio of 5:1 and were washed once with fusion buffer. Then they were resuspended in fusion buffer at a total cell concentration of $1-7x10^7$/ml. 200μl of the cell suspension was placed into a fusion chamber. The chamber was then spun in the centrifuge at 120xg for 3-4min to allow the cells to form a pellet. While the centrifuge continued to rotate, two consecutive electrical field pulses were delivered to the cells. After pulsing, the centrifugation was maintained for a further 5 minutes. Then the pulsed cells were transferred to cell culture medium. Finally the pulsed cells were plated in 96-well culture plates with $1.5x10^5$ cells in 100μl culture medium being placed in each well.

D.6. Growing hybridoma cells

The fused cells were plated in 96-well flat-bottomed plates; mouse red blood cells were added as feeders ($2x10^6$ per well). About 48 hours later, 100μl cell culture medium supplemented with HAT (hypoxanthine, aminopterin and

thymidine) were added to each well. The cells were then fed every two days with the same medium. When all SP2/o cells in control wells were dead, the cell culture medium was changed to a medium with supplemental HT (hypoxanthine and thymidine). About 8-12 days later when sufficiently grown, the culture supernatants were screened for specific antibody by CELISA (see above).

D.7. Collection of hybridoma supernatant

About ten days after fusion, 25μl of supernatant from each well with hybridoma growth was taken and tested against the immunogen cells using the CELISA assay described above. The positive hybridomas were transferred to 24-well plates for expansion. After further testing, cultures with interesting specificities could be either cloned, or the cells frozen pending more rigorous testing.

Chapter 5

RESULTS

A. IMMUNE ABSORPTION OF DP MOLECULES

The purpose of these experiments was to devise a method for extracting HLA-DP molecules from cell lysates. The concept was to use a monoclonal antibody against an HLA-DP monomorphic determinant as a capture antibody, and to have it bound to metallic beads. Since such beads can be manipulated with ease with the help of a magnet, this would make the whole procedure quite rapid.

A.1. Choice of antibodies for coating metallic beads

At the time that this work was begun, there were two monoclonal antibodies available locally. One was NFLD.M58 that binds to DP polymorphic epitopes; the other one was NFLD.M67 that appeared to be specific for HLA-DP monomorphic determinants. The problem was to choose one of them as a capture antibody, which would not block the fluorescent antibody, I3, that we had selected for detection of DP molecules by immunofluorescence.

a. The optimal concentration of anti-DP antibodies for binding cells

Before testing to see if M58 and/or M67 block the binding of I3 to HLA-DP determinants, the optimal concentrations of M58 and M67 needed to coat a given number of transfectants had to be determined. Various concentrations of M58 and M67 were tested for their reaction with a certain number of DP transfectant cells. The cells were first incubated with anti-DP antibody, then with secondary antibody conjugated with FITC. The antigen-antibody reaction between DP transfectants and M58 or M67 was estimated by quantifying fluorescence using flow cytometry. The results of M58 are shown in Figure 1; the results of M67 in Figure 2. In both figures, fluorescence increased as the dilution of antibody decreased, up to and including the original, undiluted supernatant. Beyond this, concentrating the original supernatant is seen to have little effect on the fluorescence intensity. This suggests that the original or slightly concentrated supernatant contains antibody around a concentration which causes maximum reaction between M58 or M67 and DP transfectant cells. Therefore the original (undiluted and unconcentrated) or concentrated supernatant was chosen for cross blocking tests.

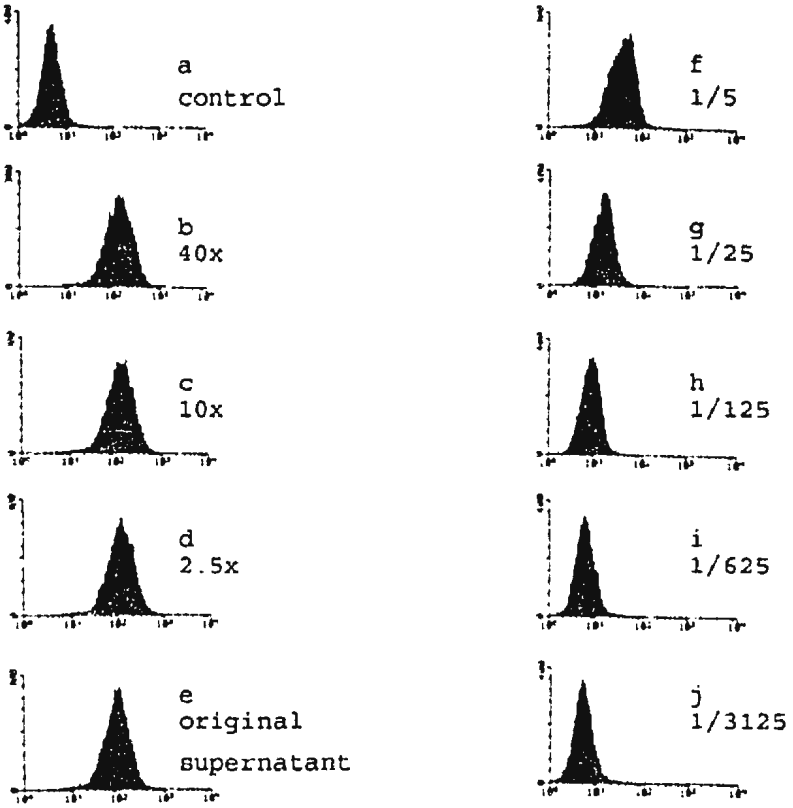
Note: Because of the design of the concentrator, it is difficult to obtain identical concentrations each time the concentrator is used.

Figure 1. Determination of the optimal concentration of M58

Various concentrations of M58 were reacted with a standard number of L25.4 transfectant cells ($2x10^5$). The reaction was identified by a second antibody labelled with FITC and was quantified by flow cytometric analysis of fluorescence. A. Panel *a* shows the negative control in which the cells had not been exposed to the FITC-labelled antibody. In panels *b-d*, concentrated M58 supernatants were used; in panel e, original M58 hybridoma supernatant was used; in panels *f-j*, M58 was serially diluted. B. The fluorescence intensity* increased as the concentration of M58 increased. Beyond the concentration of 2.5x, the increase in fluorescence intensity became less.

* Fluorescence intensity refers to the mean intensity.

A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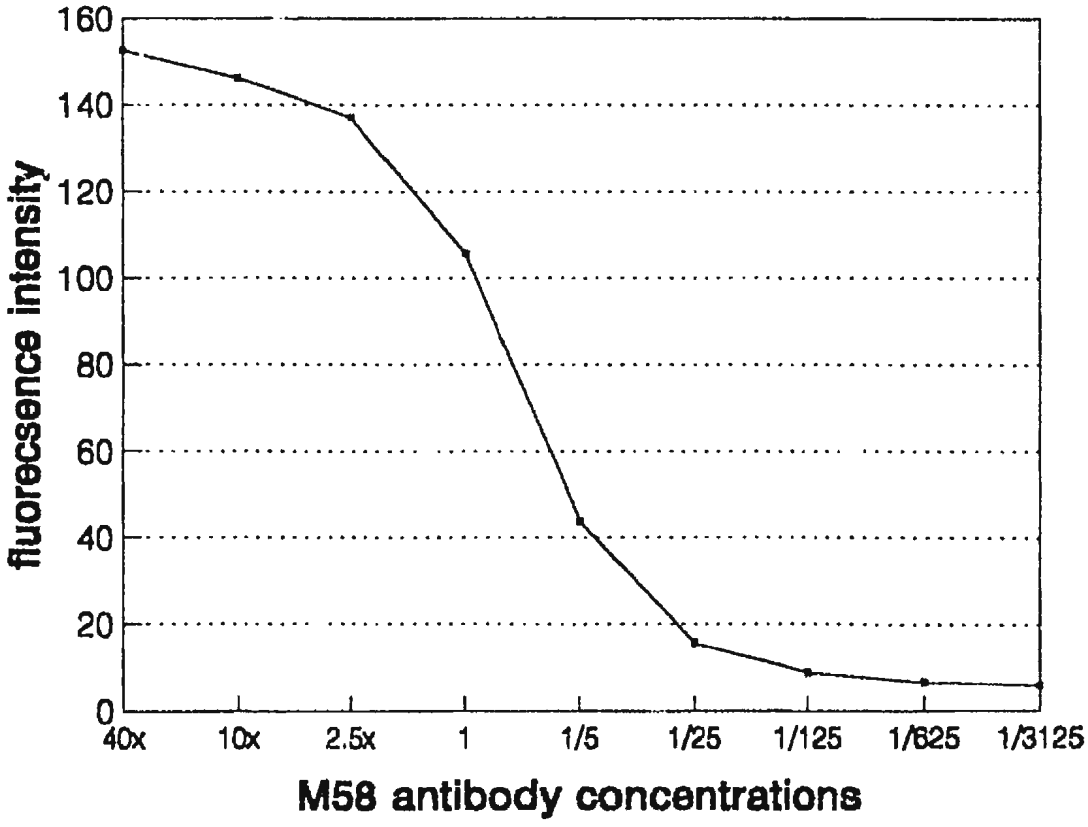
B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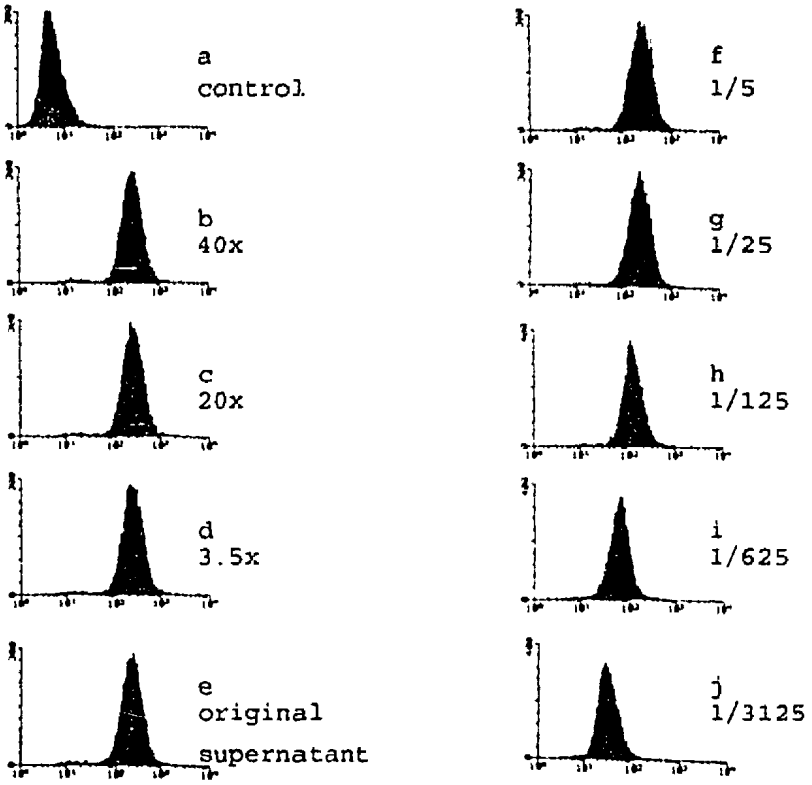
1/625
1/3125
M58 antibody concentrations

Figure 2. Determination of the optimal concentration of M67

Various concentrations of M67 were reacted with a standard number of L25.4 transfectant cells ($2x10^5$). The reaction was identified by a second antibody labelled with FITC and was quantified by flow cytometric analysis of fluorescence. A. Panel *a* shows the negative control in which the cells had not been exposed to the FITC-labelled antibody. In panels *b-d*, different concentrations of original M67 supernatant were used; in panel *e*, original M67 hybridoma supernatant was used; in panels *g-j*, M67 was serially diluted. B. The increase in fluorescence intensity* became less apparent as M67 concentration continued increasing.

* Fluorescence intensity refers to the mean intensity.

A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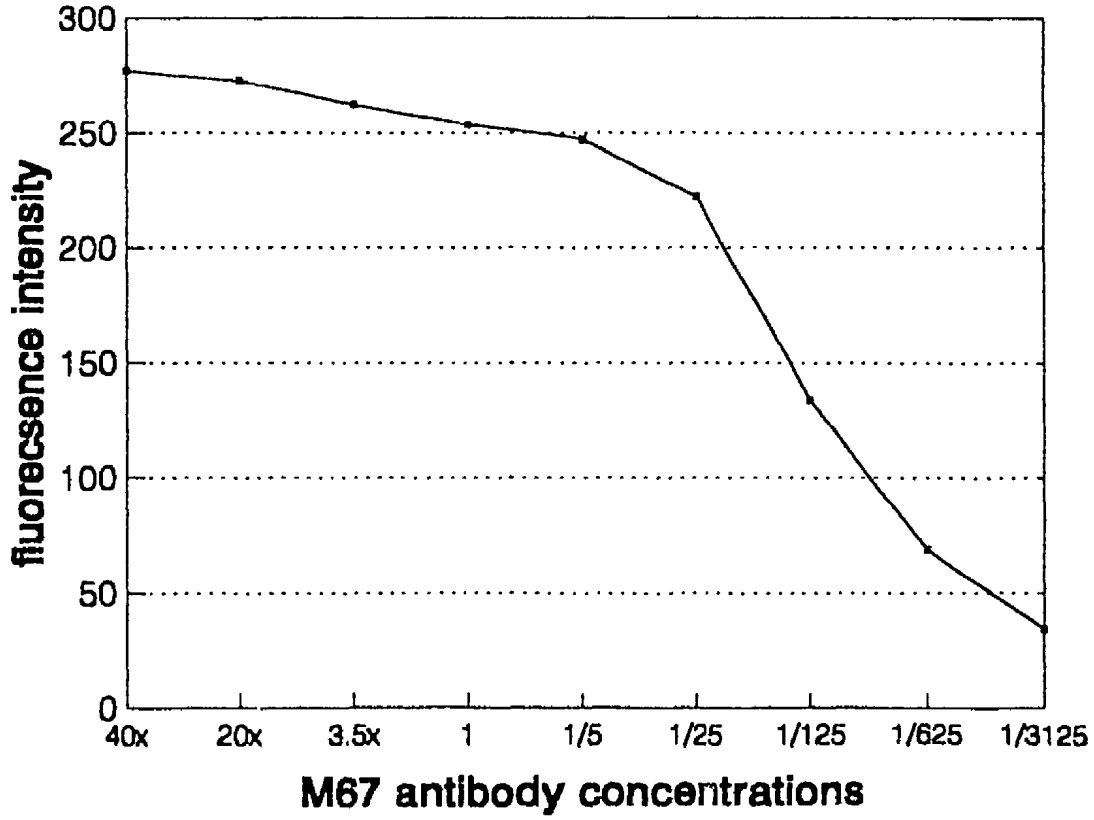
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M67 antibody concentrations

b. Cross blocking I3 by M58 or M67

An experiment for testing M58 and M67 was performed to see if those antibodies would block I3 binding to DP transfectants. The cells were first incubated with M58 or M67, then with I3. After that, the cells were examined for fluorescence using flow cytometry. The results are shown in Figure 3 , in which we can see that, after incubation with M67, the DP transfectants could still be stained by I3, and the fluorescence did not change, as comparing histogram *d* with *b* and *c*; while after incubation with M58, the transfectants showed greatly reduced fluorescence, as comparing histogram *e* with *b* and *c*. From these results, we can conclude that M67 does not block I3 binding to DP. Monoclonal antibody M67 can therefore be used as a capture antibody for absorption of DP molecules, so that I3 can be used for demonstrating the presence of these molecules on the beads. M58 completely blocks I3 binding to DP. This property can be used in this study for an indirect (blocking) demonstration of absorbed DP molecules.

c. Blocking nonspecific fluorescence when using the metallic beads

In this study, one problem with the use of FITC-labelled I3 to demonstrate absorbed DP molecules was nonspecific fluorescence caused by direct binding of I3 to

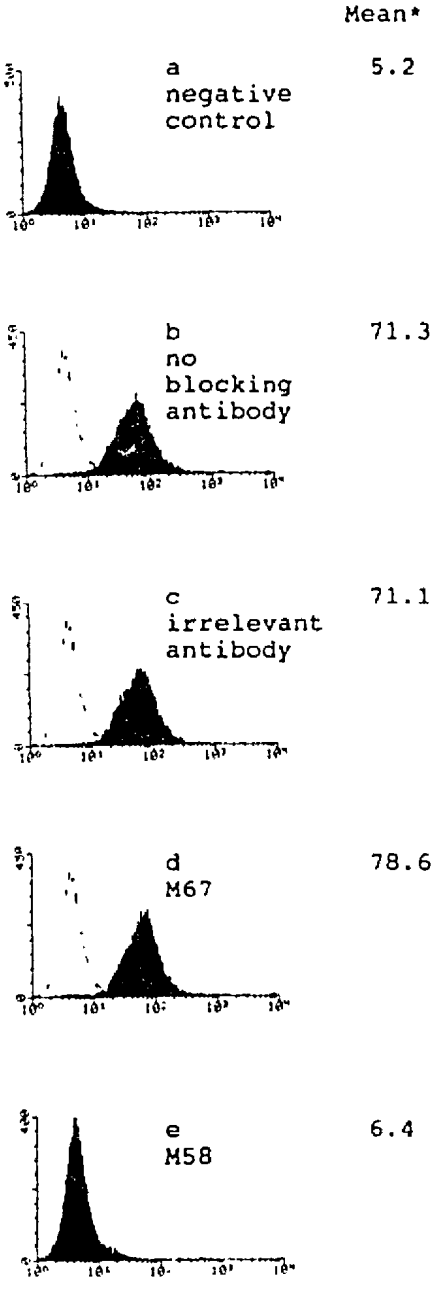

Figure 3. Cross blocking test

M67 and M58 were tested to see if they block fluorescent antibody I3 binding to DP transfectant cells. Panel *a* shows the cells that have not been exposed to the fluorescent antibody I3; panel *b* shows the positive control in which no blocking antibody is added; panel *c* serves as another positive control in which an irrelevant antibody is added before the fluorescent antibody I3 reacts with the cells; panel *d* shows that pre-incubation of M67 with the transfectant cells does not interfere with the binding of I3 to the cells, while panel e shows that the pre-incubation of M58 with the cells causes a failure of I3 to bind to the cells.

* Mean fluorescence intensity

metallic beads. This occurred because I3 is a mouse IgG antibody and the metallic beads used are coated with a goat polyclonal antibody against all mouse IgG subclasses. To overcome this problem, affinity purified normal mouse IgG as well as normal mouse serum were tested respectively in serial dilutions for their ability to block nonspecific fluorescence. The metallic beads, after coating with anti-DP antibody, were incubated with purified normal mouse IgG or normal mouse serum, so that the unoccupied binding sites of goat immunoglobulins were filled with normal mouse IgG. Then the metallic beads were stained with I3 and examined for fluorescence using flow cytometry. The results are shown in Figure 4 (purified normal mouse IgG was used as blocking agent) and Figure 5 (normal mouse serum was used as blocking agent). Figure 4 shows that the metallic beads without being blocked had strong fluorescence, which is represented by histogram *b*. When purified mouse IgG was added, the fluorescence was blocked. As the amount of blocking IgG was reduced, the fluorescence gradually increased. The same effect can be seen in Figure 5. As normal serum was diluted, the fluorescence increased, indicating that the block became less complete. These results tell us that normal mouse serum or purified mouse IgG are effective for blocking nonspecific fluorescence. In all later experiments the beads were always incubated in mouse serum before I3 antibody was added.

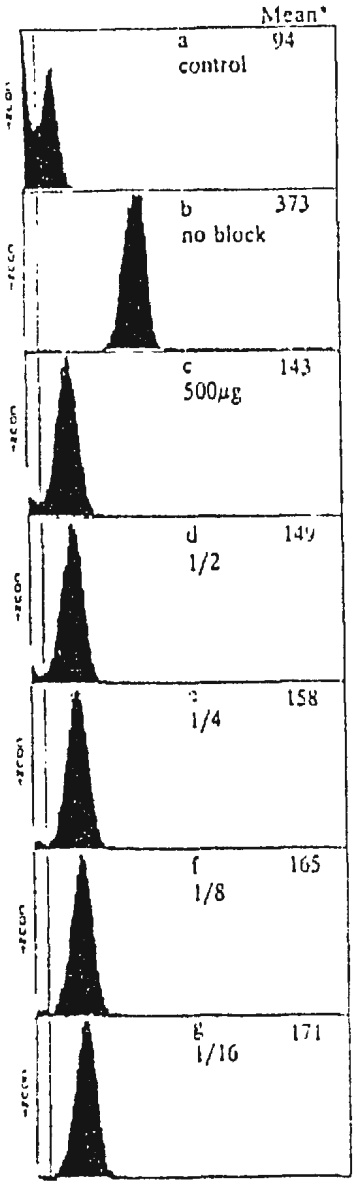

Figure 4.Inhibition of non-specific staining using normal mouse IgG

This shows how the antibody-coated metallic beads take up FITC-labelled I3 (anti-DP) non-specifically even when there are no DP molecules on the beads (panel *b*). Panel *a* shows the negative control beads which have not been exposed to FITC. Panels *c-g* show how various dilutions of normal mouse IgG, added to the beads before I3, inhibit this non-specific binding completely. The amounts of IgG added were 500µg(*c*), 250µg(*d*), 125µg(*e*), 62.5µg(*f*) and 31.25µg(*g*).

* Mean fluorescence intensity

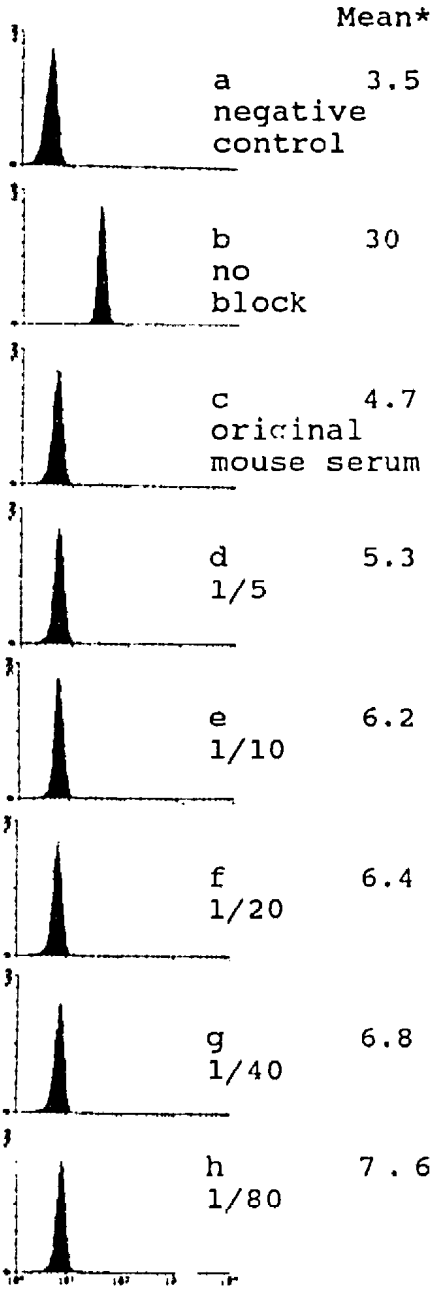

Figure 5. Inhibition of non-specific staining using normal mouse serum

Normal mouse serum was used to block nonspecific staining of metallic beads by FITC-labelled I3. Panel *a* shows the negative control beads which have not been exposed to FITC. Panel *b*, as in figure 4, shows nonspecific binding between I3 and metallic beads. Panels *c-h* show how various dilutions of normal mouse serum, added before I3, inhibit this nonspecific binding effectively.

* Mean fluorescence intensity

A.2 Absorption of DP molecules from cell lysates

The procedure to obtain purified membrane molecules using affinity chromatography is finicky and time-consuming. In this study, we attempted to use metallic beads coated with antibody against an HLA-DP monomorphic determinant for absorption of DP molecules from cell lysates, to simplify the purification procedure.

HLA-DP absorption was done by mixing metallic beads, coated with anti-DP antibody, with a cell lysate, followed by incubation overnight at 4°C. After that, the beads were examined for DP molecule absorption by staining with the fluorescent antibody I3. The DP-absorption was estimated by quantifying the fluorescence using flow cytometry. As we did not have any knowledge about the cell numbers to be used for preparing cell lysates, or the amount of beads to be used for absorption, two experiments were done.

In the first experiment, the effect of increasing the number of cells in the lysate was studied. Using a standard number of beads($1x10^6$), absorptions were done in lysates made from $1x10^5$, $5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$ and $1.5x10^7$ B cell line cells. As a control, the absorption was also done in a lysate made from $1x10^6$ normal mouse lymphocytes. One million metallic beads coated with an irrelevant antibody were used as another control. The results are shown in Figures 6a and 6b. It can be

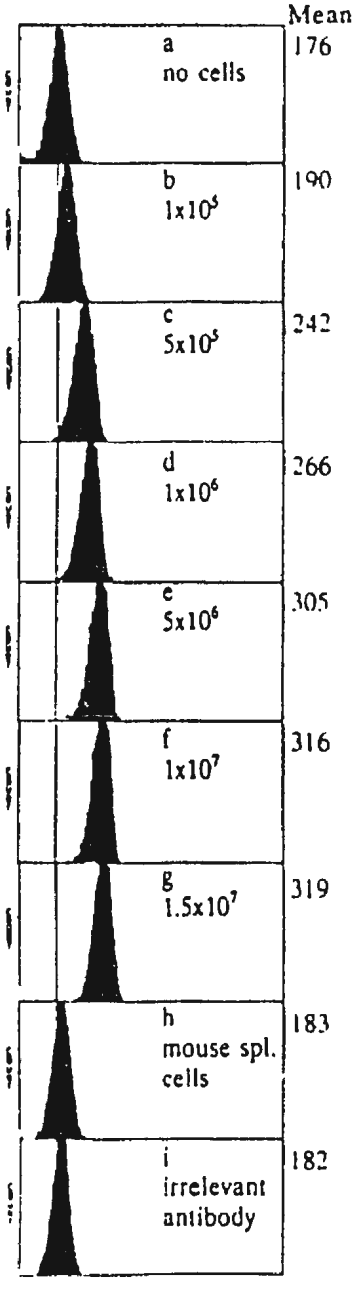

Figure 6-a. Serial extraction of DP molecules using a stantard number of beads

This experiment indirectly shows the uptake of DP molecules by a standard number of metallic beads ($1x10^6$) when they were placed in a standard lysate volume but made by lysing different numbers of cells. Panel *a* shows the negative control where the metallic beads had not been exposed to DP molecules. In panels *b-g* the number of cells (HO301 B cell line) lysed in each case are shown. Panel *h* is the result of using a mouse spleen cell lysate (10^6 cells) as a control. Panel *i* used an irrelevant coating antibody on the beads, which were then exposed to a lysate containing 10^6 HO301 B cell line cells. The fluorescent antibody used to reveal bound HLA-DP molecules was I3.

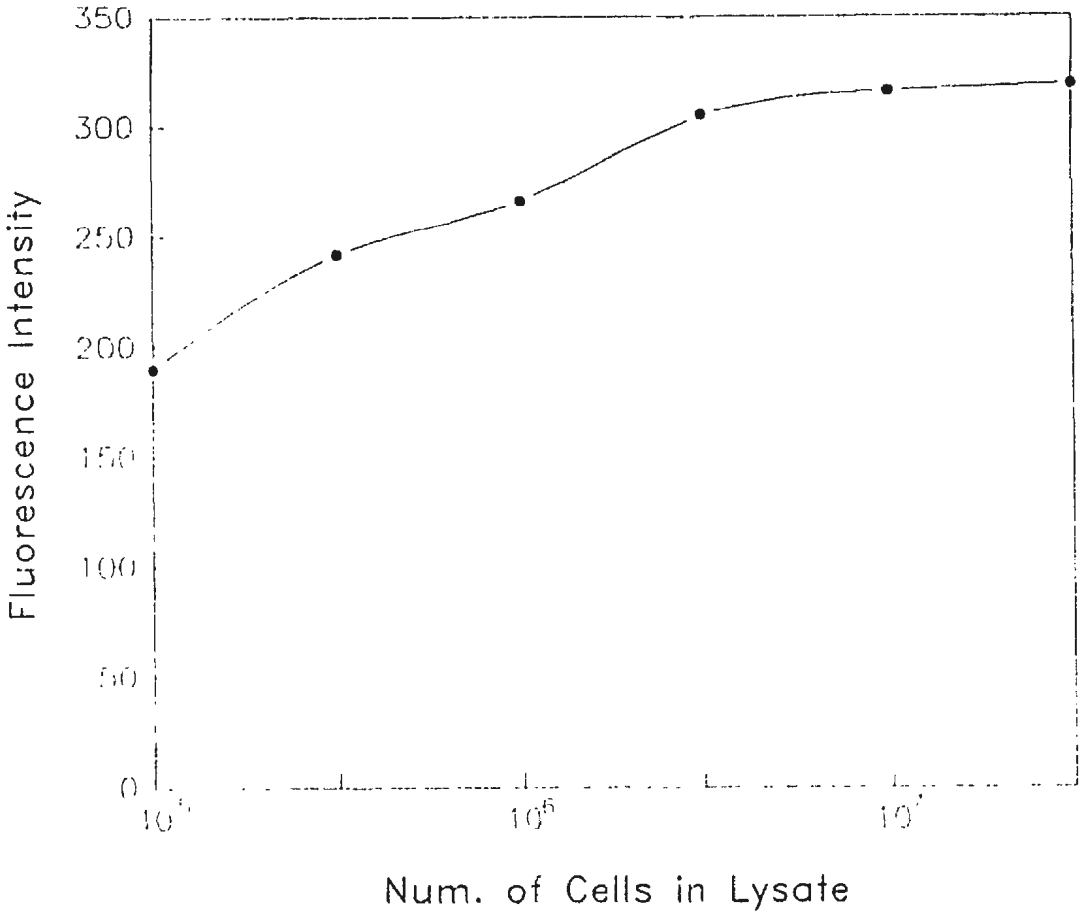

Figure 6-b. Serial extraction of DP molecules using a standard number of beads (1 million)

The absorption of DP molecules in lysates made of different numbers of cells were plotted. It appears that, as the cell number reached $5x10^6$, the increase of absorption became less significant with further increases in cell number.

seen , from histogram *b* to *g*, that as the cell number in the lysate was increased, the absorption also increased. It is obvious in histogram *h* and *i* that the beads coated with anti-DP antibody picked up very little in the mouse cell lysate and the irrelevant antibody coated beads also picked up almost nothing from the B cell line lysate.

In the next experiment, two serial extractions from a lysate made from a standard number of B cell line cells ($1x10^6$) were performed, one with $2x10^6$ metallic beads coated with anti-DP antibody, the other with $2x10^7$ metallic beads coated with the same antibody. After five extractions, the DP-absorptions were compared between the two groups. The results are presented in Figure 7. It can be seen from the change in the fluorescence intensity that the lysate made from $1x10^6$ cells allowed 2 million beads to do an effective extraction all 5 times. In order to have a reasonable density of DP molecules on each bead, 20 million beads seem to be too many for a lysate made from $1x10^6$ cells.

A.3 Indirect demonstration of absorbed molecules

In order to identify DP molecules absorbed onto metallic beads more specifically, the following experiment was done. It was already known from a previous experiment that M58

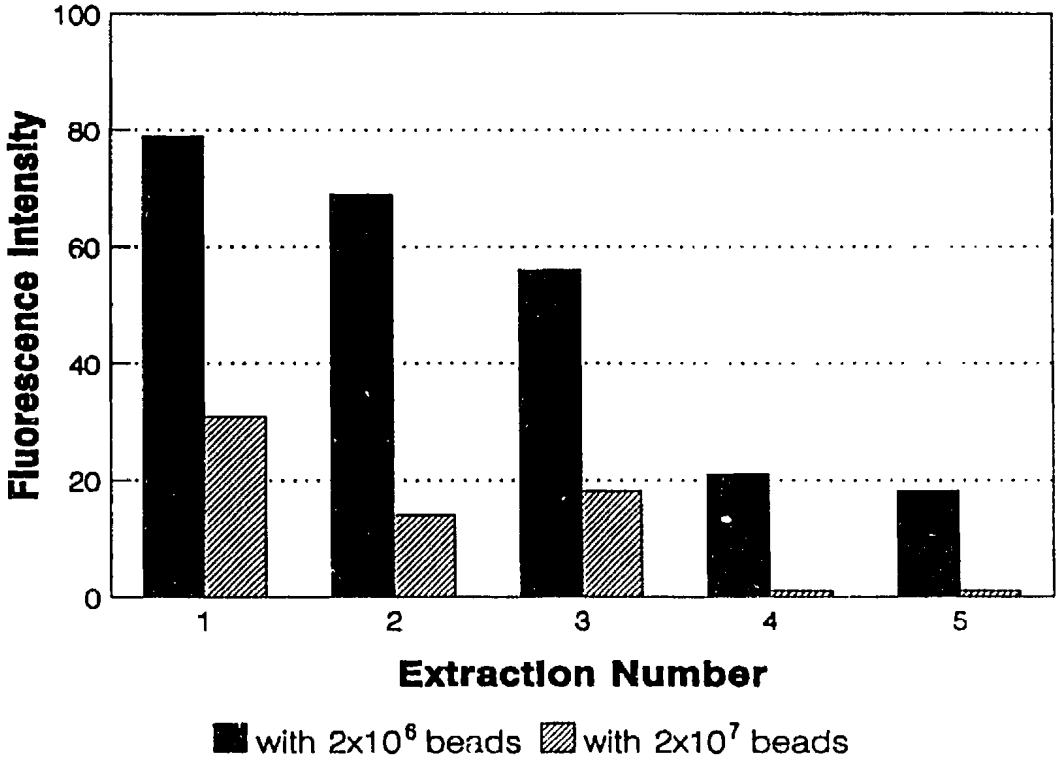

Figure 7. Serial extraction of DP molecules in lysates prepared with a standard number of cells

This diagram shows the average amount of HLA-DP per extraction, assessed by FITC-labelled I3, when a standard lysate of HO301 B cells (10^6 total) was serially extracted by antibody coated beads. The solid bars show the result when 5 serial extractions were made using $2x10^6$ beads each time. The hatched bars show the result when $2x10^7$ beads were used for each extraction. Fluorescent intensities represent values from which the background (negative control) intensities have been subtracted.

could effectively block I3 binding to HLA-DP. Since M58 is specific for DP polymorphic epitopes, the molecules blocked by it must be HLA-DP.

The WT51 B cell line is homozygous for DPB1*0201, which is within the antigen repertoire of the M58 antibody. In this experiment, this cell line was used for preparing a cell lysate. The absorption of DP molecules was performed as described before. After that, the beads were blocked by incubating with M58. Then they were stained with I3. The fluorescence was examined using flow cytometry. The results are shown in Figure 8, in which we can see a diminished fluorescence when absorbed beads were blocked by M58. This indicates that metallic beads had absorbed DP molecules onto them.

A.4. Demonstration of absorbed DP molecules by biotin-M67

To identify DP molecules absorbed onto beads, another experiment was done, in which M58 was used as a capture antibody coated on beads and M67 labelled with biotin served as an indicator. M67 is known to be reactive with DP molecules but not with DR or DQ (on transfectants). Again, the WT51 B cell line was used for preparing a cell lysate. After immune absorption, the beads were stained by M67-biotin-avidin-FITC

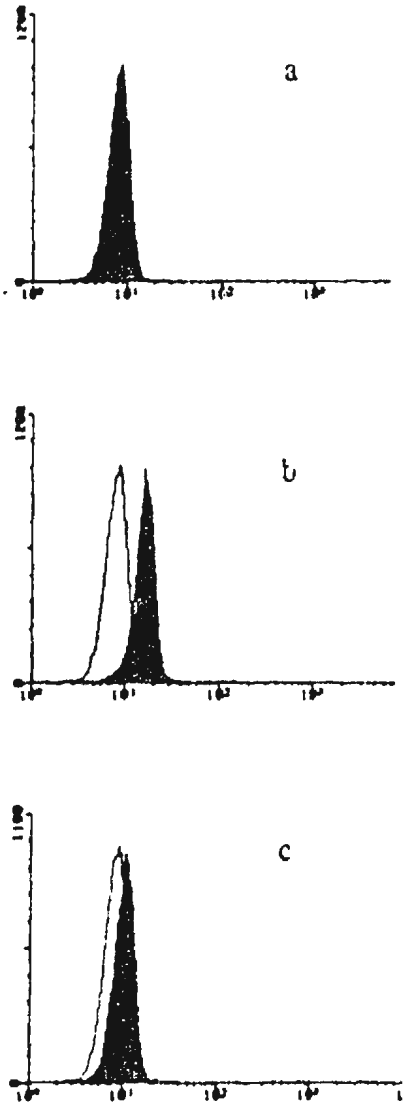

Figure 8. Specific blocking test

A specificity test demonstrates that the FITC-labelled I3 is binding to HLA-DP molecules. Panel *a* is a negative control showing the staining with I3 of beads that had not been exposed to DP molecules. Panel *b* is the positive control where DP coated beads were stained with FITC-labelled I3. Panel c shows the reduction of fluorescence when unlabelled M58 (anti-DP antibody) was added to the beads before the addition of FITC-labelled I3.

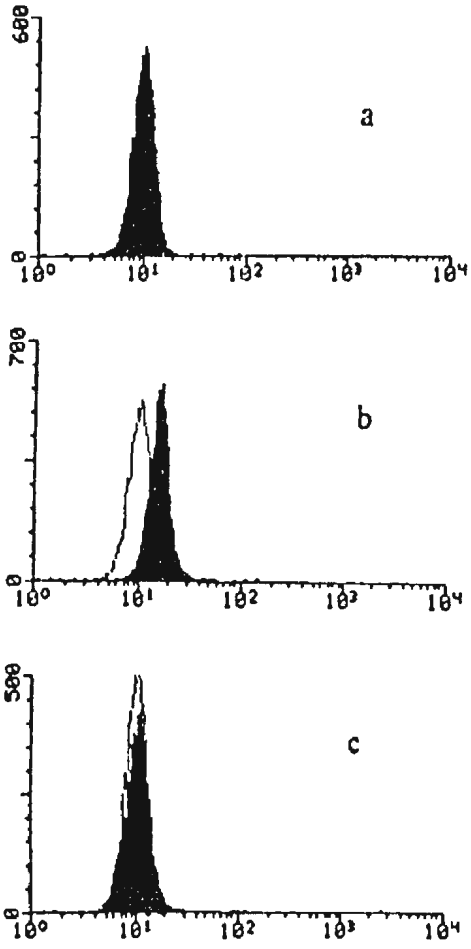

Figure 9. Demonstration of DP by biotin-M67

The absorbed molecules on metallic beads were identified by biotinylated M67. Panel *a* shows the negative control beads; panel *b* shows the beads absorbed in a WT51 cell lysate which contains DP molecules; panel *c* shows the beads absorbed in a cell lysate (made from T cell line cells) that does not contain DP molecules. Only the beads absorbed in the DP-containing cell lysate were stained by M67-biotin-avidin-FITC.

system. The results are shown in Figure 9. It can be seen that the beads absorbed in a WT51 cell lysate showed a distinct binding to biotinylated M67. This indicates that DP molecules were absorbed onto metallic beads.

B. IMMUNOGENIC PROPERTIES OF BEAD-PURIFIED MOLECULES

Using antigens attached to a carrier (like nitrocellulose membrane, glass or agarose beads) to immunize mice has proven to be effective. In this study, we attempted to use HLA-DP molecules absorbed onto metallic beads as an immunogen to induce an antibody response to HLA-DP in the mouse.

B.1 Antigen dose response

In order to establish a reasonable dose of antigen for use as an immunogen, two experiments were performed initially. Metallic beads loaded with DP molecules were injected in different doses into three groups of three mice each. The serum antibody responses were measured using cellular enzyme-linked immunoassay (CELISA). The two experiments involved two different cell lines and somewhat different loading conditions for the beads.

The first experiment used the COX B cell line (see Materials and Methods). A lysate containing $8x10^7$ COX cells in 1.5 ml was used to load $2x10^8$ beads by the method described earlier. Antigen loaded beads were injected subcutaneously into mice, along with complete Freund's adjuvant (CFA, see Materials and Methods), as follows: 3 mice received 2 million beads each; 3 mice received 10 million beads each and 3 mice were injected with 50 million beads each. Eighteen days following the primary immunization, tail-bleeding was done and the sera were tested against COX cells using the CELISA method. The results in Figure 10 show a clear dose response; for example, at the 1/24 dilution, the optical densities were 50 million beads > 10 million beads > 2 million beads > non immune serum.

The next experiment was essentially the same except that the SAVC cell line was used and the lysate this time contained 10^8 cells in 1.5 ml. Figure 11 shows the same results as in Figure 10, the dose of 50 million beads caused a better antibody response than 10 million beads and 2 million beads.

The conclusion from these experiments is that 50 million beads are more effective than are smaller numbers, for evoking an antibody response in the mouse.

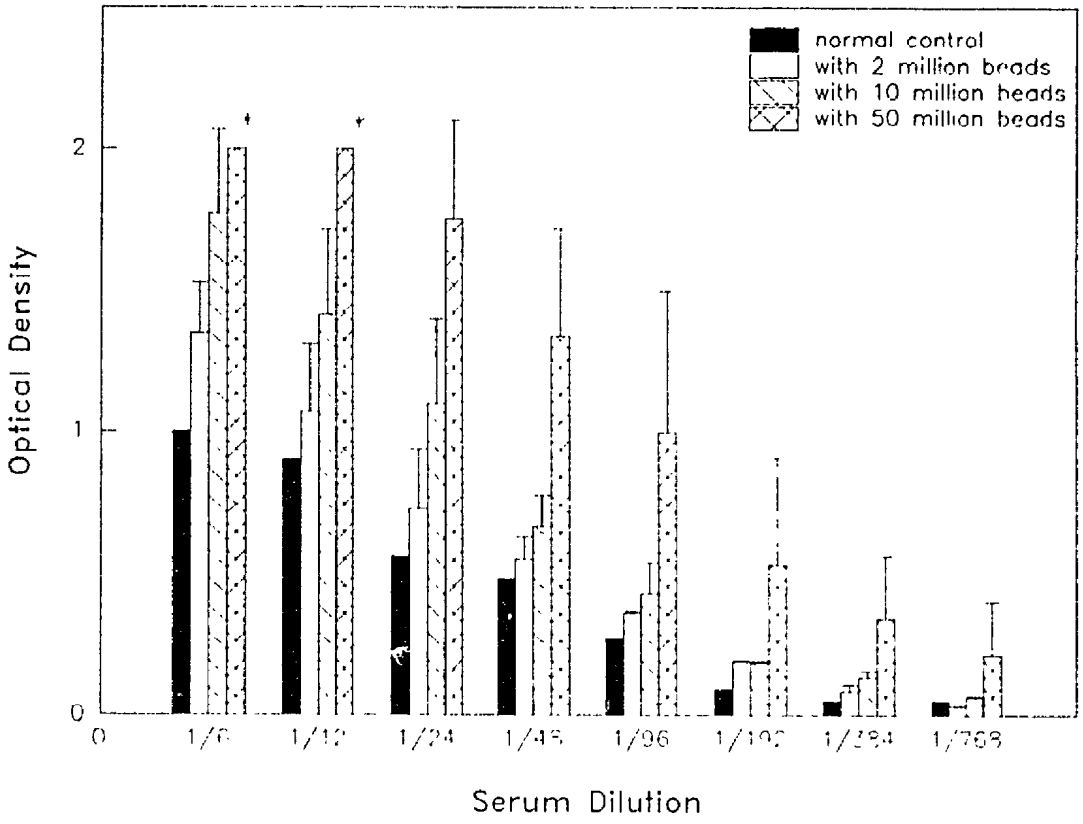

Figure 10. Immunogen dose response

Data from an immunogen dose response determined by injecting Balb/c mice with different numbers of metallic beads that had been loaded with DP molecules from a lysate made from the COX B cell line. Eighteen days after the primary immunization, the sera were tested using the CELISA method.

* The optical density of these dilutions exceeds 2.00.

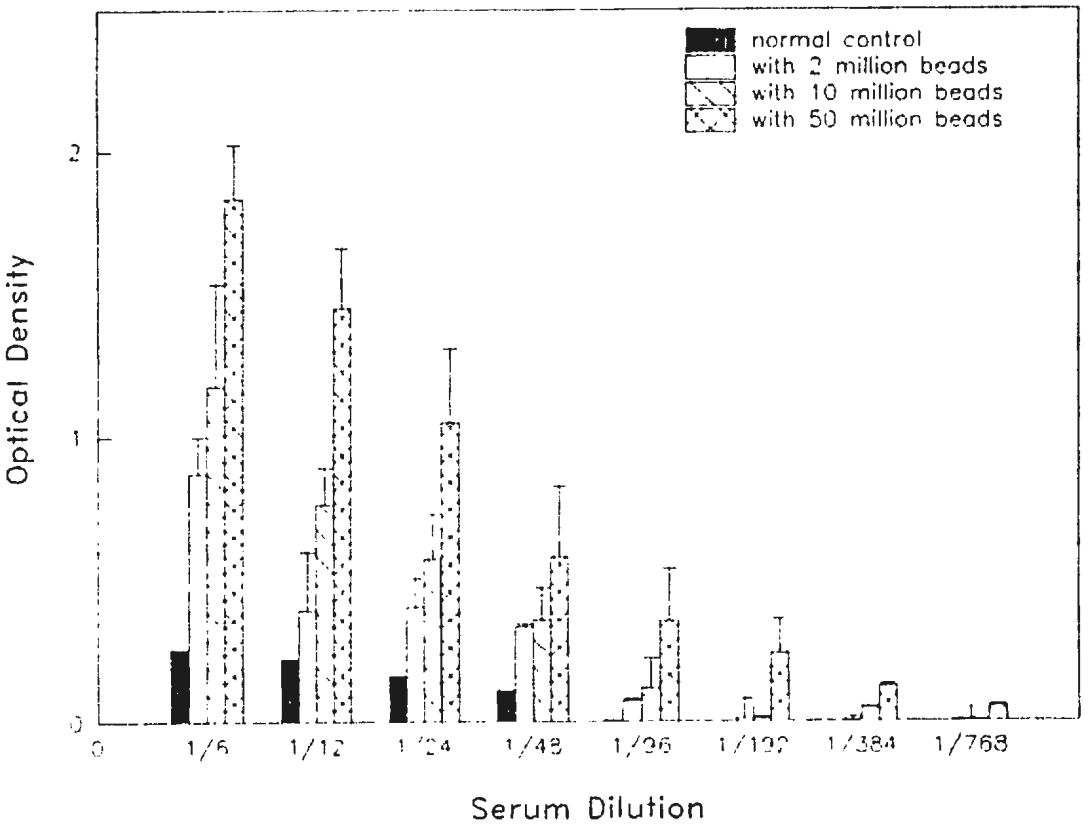

Figure 11. Immunogen dose response

This figure represents the same experiment as in figure 10 except that the metallic beads were loaded with DP molecules from SAVC B cell line.

B.2. Antibody response following the secondary immunization

Again, two experiments were performed, with different cell lines, to examine the secondary antibody response.

In the first experiment, HO301 B cell line was used (see Materials and Methods). 4-6 weeks following the primary immunization that was done through subcutaneous injection of 50 million DP loaded beads with CFA, the secondary immunization was performed by intraperitoneal injection of the same amount of beads with incomplete Freund's adjuvant. Three mice were done; one died of injury after the secondary injection. The sera were obtained through tail-bleeding one week after the secondary immunization and tested against HO301 cells using the CELISA method. The results are shown in Figure 12, in which a strong antibody response can be seen; at 1/48 dilution, the OD value is still higher than 2.

The second experiment using the WT51 B cell line was performed under the same conditions. The sera from three mice were diluted from 1/50 to 1/6400 for testing. Figure 13 shows that, at 1/1600 dilution, the antibody response was still distinctive.

These results further indicate that DP loaded metallic beads are highly immunogenic.

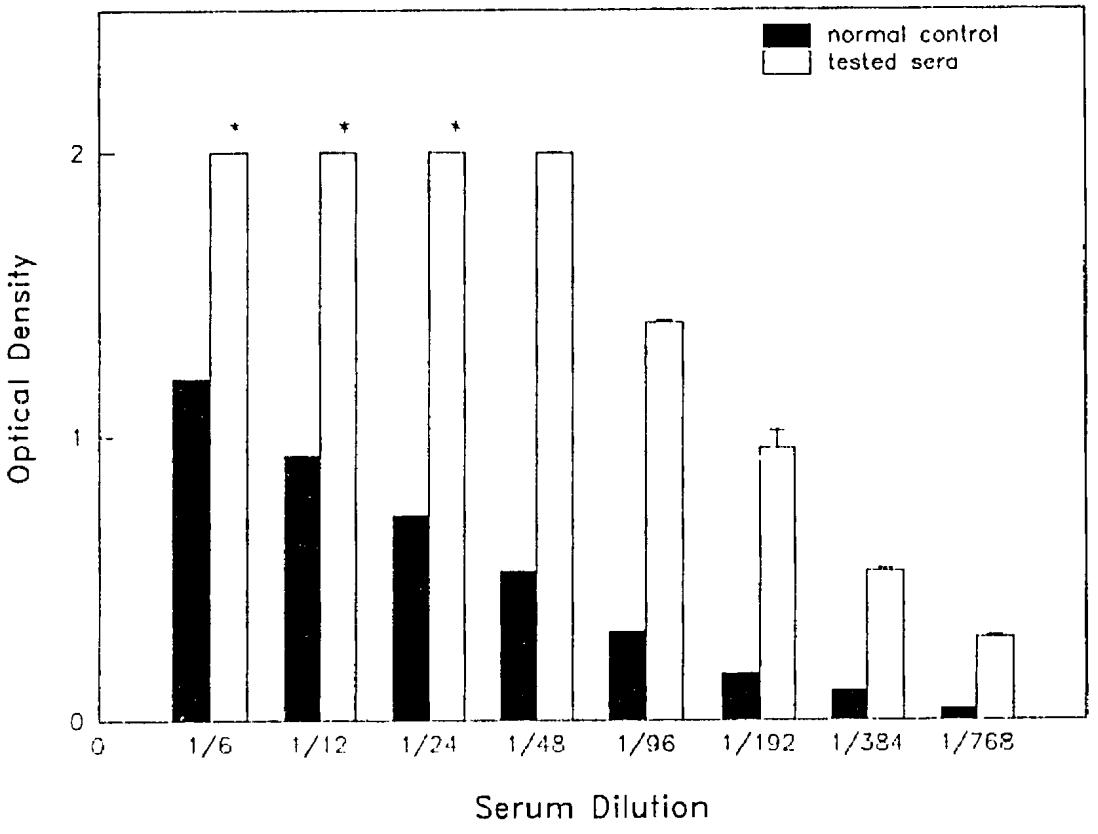

Figure 12. Secondary antibody response

The secondary antibody response was determined on Balb/c mice one week following the second immunization with $5x10^7$ metallic beads loaded with DP molecules from a lysate made from HO301 B cell line cells.

* The optical density of these dilutions exceeds 2.00.

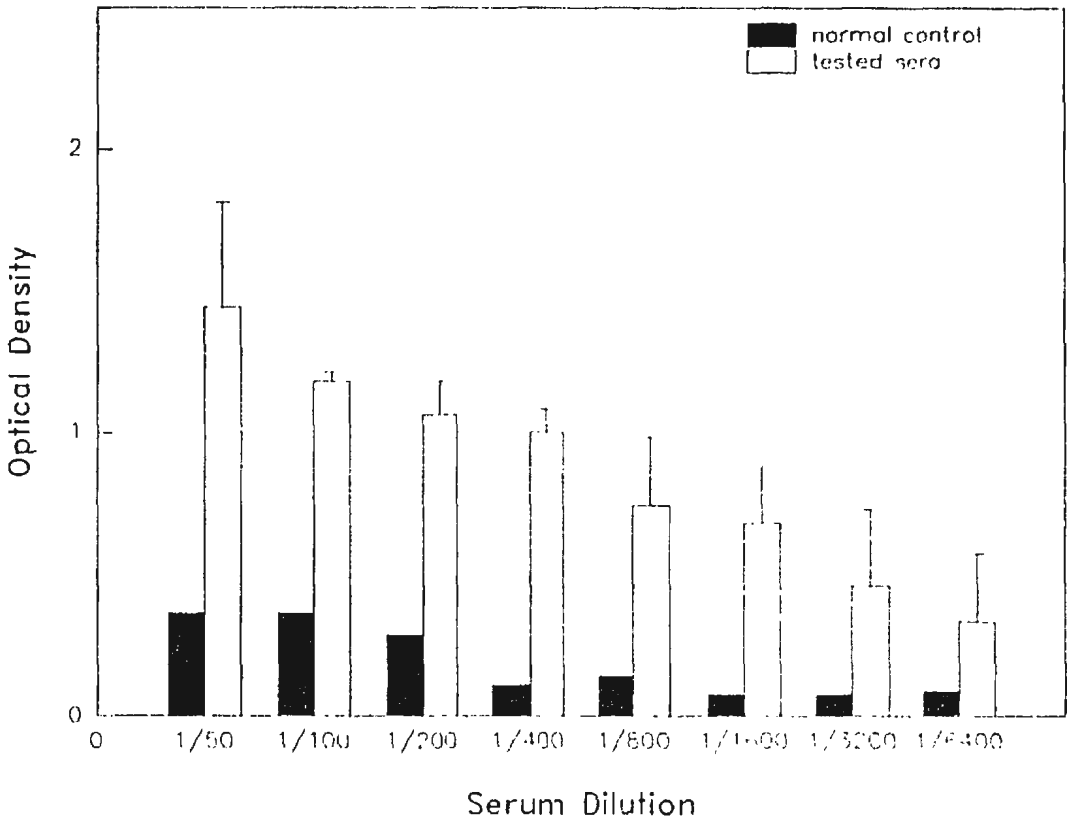

Figure 13. Secondary antibody response

The secondary antibody response was determined on Balb/c mice one week after the second immunization with $5x10^7$ metallic beads loaded with DP molecules from a lysate made from WT51 cells.

B.3. Comparison of beryllium sulphate and CFA, using DP molecules from L-cell transfectants as immunogen

Beryllium sulphate is an alternative adjuvant, which has the interesting property of driving the immune response to produce IgG2 antibodies; this contrasts with complete Freund's adjuvant which tends to result in IgG1 antibodies. The former, which activate complement are more useful for some purposes. In order to study its efficacy in promoting antibody responses in mice, beryllium sulphate and complete Freund's adjuvant were compared. Each of two adjuvants was used in separate primary immunizations with DP-absorbed beads. The comparison was performed in two groups of mice; one used C3H mice, the other used Balb/c mice. Each group was further divided into two subgroups, one with use of beryllium sulphate, the other with use of complete Freund's adjuvant. L-cell transfectants expressing HLA-DP were used this time in an attempt to use a potentially purer source of DP molecules. Since the L-cells are of C3H origin, the use of C3H mice is the ideal for this experiment.

Two sorts of DP transfectant cells (DP8303 and DP8306) were used for preparing cell lysate. In the primary immunization, which was done through subcutaneous injection of antigen with beryllium sulphate or complete Freund's adjuvant, $3.5x10^8$ DP8303 transfectant cells were lysed in 1.5ml to load $4x10^8$ beads; $4x10^8$ DP8306 transfectant cells were lysed in

1.5ml to load $4x10^8$ beads. In each subgroup, two mice were immunized with DP8303 beads; two mice were immunized with DP8306 beads. $5x10^7$ beads were injected into each mouse. Four weeks later, the sera were tested against transfectant cells using the CELISA method. The secondary immunization was done through intraperitoneal injection of the same dose of DP loaded beads with incomplete Freund's adjuvant. One week later, the secondary antibody response was estimated using the CELISA method.

The serum testing after the primary immunization did not show any significant antibody response in any group of mice. However, the secondary antibody response was obvious. The comparison of beryllium sulphate and complete Freund's adjuvant is given in Figure 14 (in C3H mice) and Figure 15 (in Balb/c mice). It can be seen that, in both groups, the antibody response in the mice with use of complete Freund's adjuvant was higher than in the mice with use of beryllium sulphate. This suggests that complete Freund's adjuvant was more effective than beryllium sulphate in promoting antibody responses in the mice.

B.4. Comparison of antibody responses to L cells and to transfectants

Sera from the previous experiment were further tested using the CELISA method, in order to see how much of the

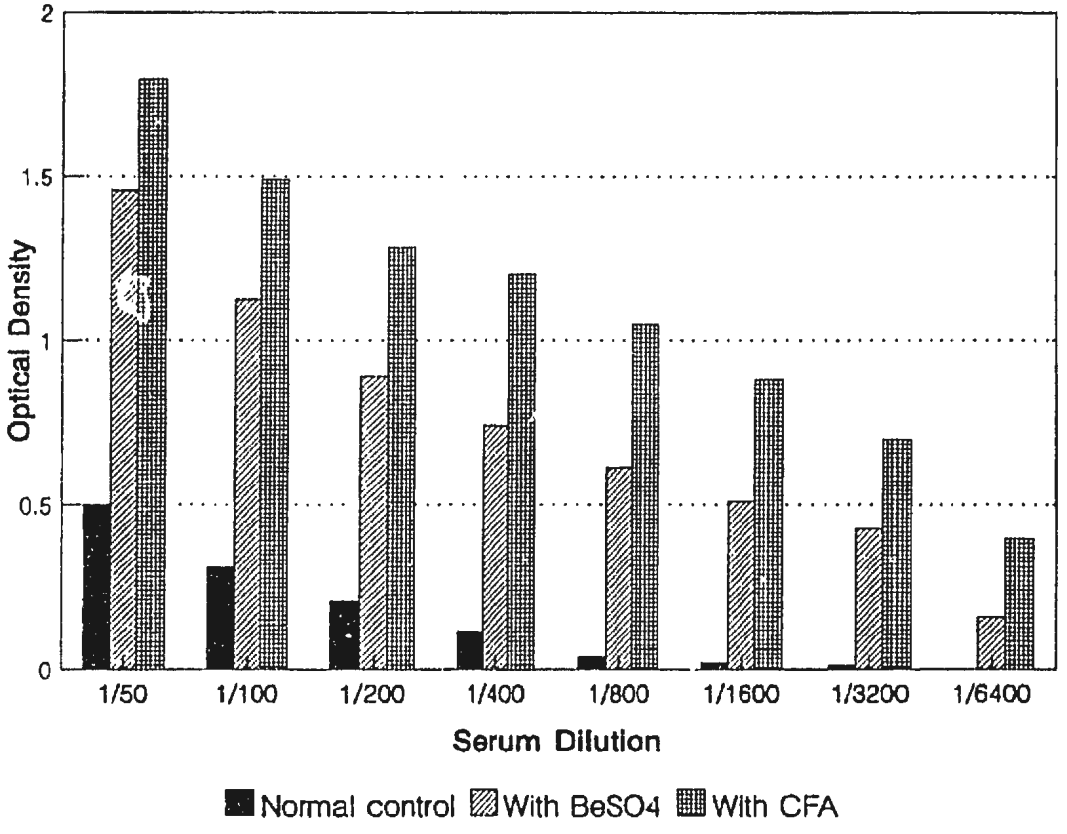

Figure 14. Comparison of BeSO4 and CFA in C3H mice

The influence of beryllium sulphate and complete Freund's adjuvant on antibody response was compared in C3H mice. In the primary immunization, both adjuvants were respectively used with metallic beads loaded with DP molecules in two groups of mice. In the secondary immunization the incomplete Freund's adjuvant was used in both groups of mice with the same dose of the beads. This diagram represents the secondary antibody response measured in both groups of mice one week after the second immunization.

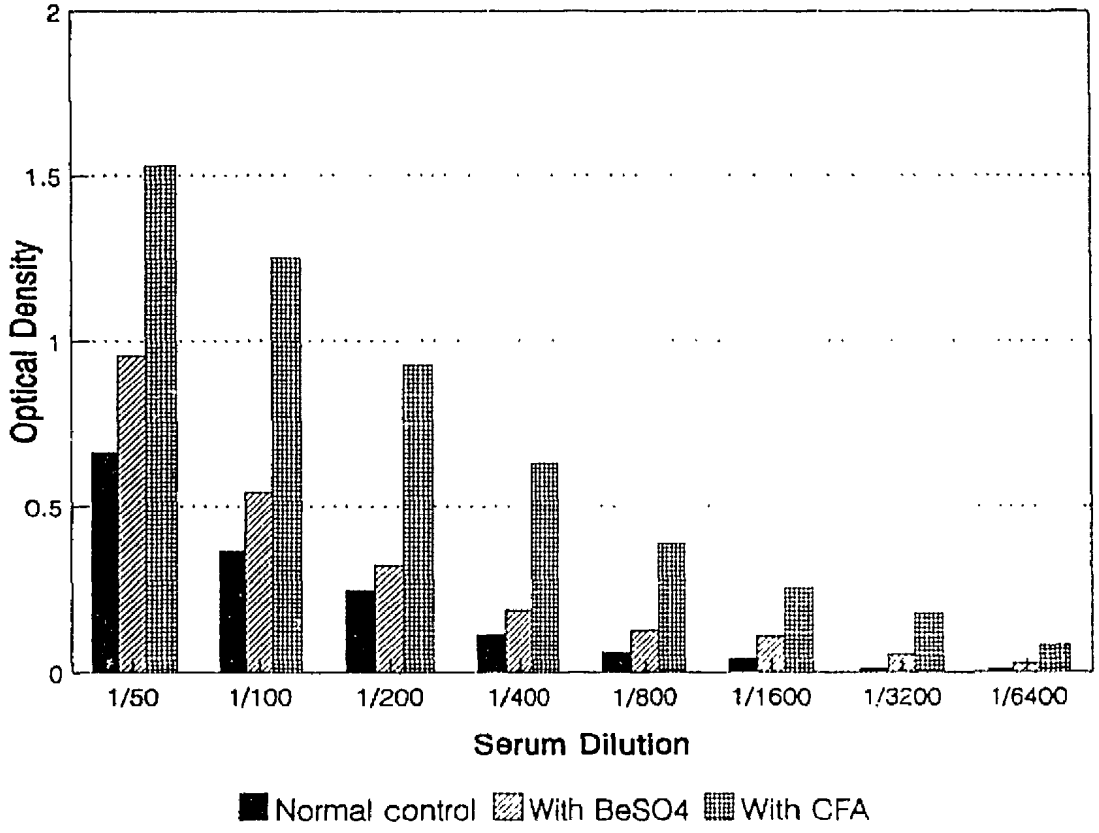

Figure 15. Comparison of BeSO4 and CFA in Balb/c mice

The influence of beryllium sulphate and complete Freund's adjuvant on antibody response was compared in Balb/c mice. The immunization protocol is as same as in Figure 14, and the diagram shows a similar pattern.

reactivity was against L cell determinants and not against the DP molecules expressed on the transfectants. This was a not unlikely situation since the L-cells are of C3H origin and half of the immunized mice were Balb/c; these mice differ at their H-2 loci, C3H being $H\text{-}2^k$ and Balb/c being $H\text{-}2^d$.

Figure 16 shows the comparison of antibody responses to L cells and to DP transfectants in C3H mice. It can be seen that there was considerable (and unexpected) reactivity to L-cells, but the reactivity against DP transfectants was superior to L cells. Figure 17 shows the same comparison in Balb/c mice. It is obvious that, in Balb/c mice, the antibody response to DP transfectants cannot be differentiated from the response to L cells. This suggests that to induce an antibody response to DP, C3H transfectant cells may be more suitable for use in C3H mice than in Balb/c mice. It also suggests that antibody coated beads could pick up things other than human class II molecules.

C. CENTRIFUGAL-ELECTROFUSION (CEF) OF IMMUNOMAGNETICALLY SELECTED SPLEEN CELLS

The production of mouse hybridomas is usually accomplished through fusion of mouse spleen cells with partner cells using the chemical fusagen, polyethylene glycol (PEG).

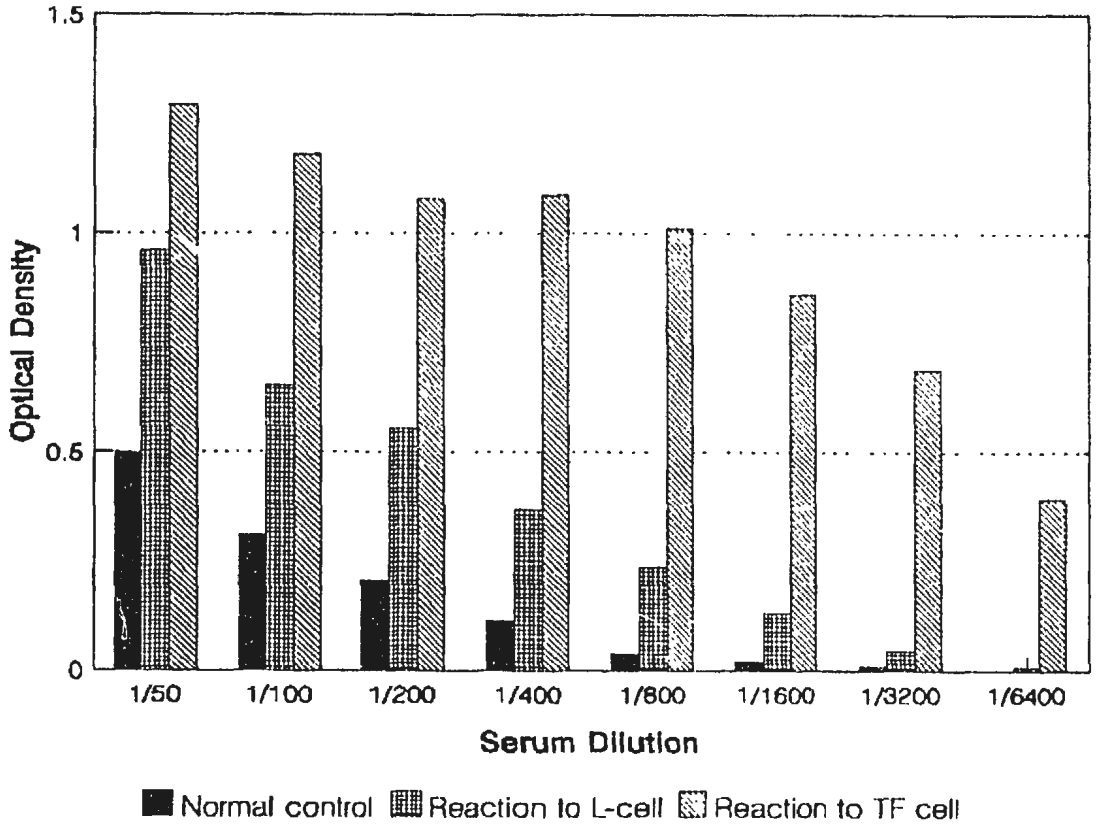

Figure 16. Serum antibody specificity in C3H mice

The antibody responses to L-cells and to L-cell DP transfectants were compared in C3H mice. As the serum was diluted, the reactivity against DP transfectants became more distinctive.

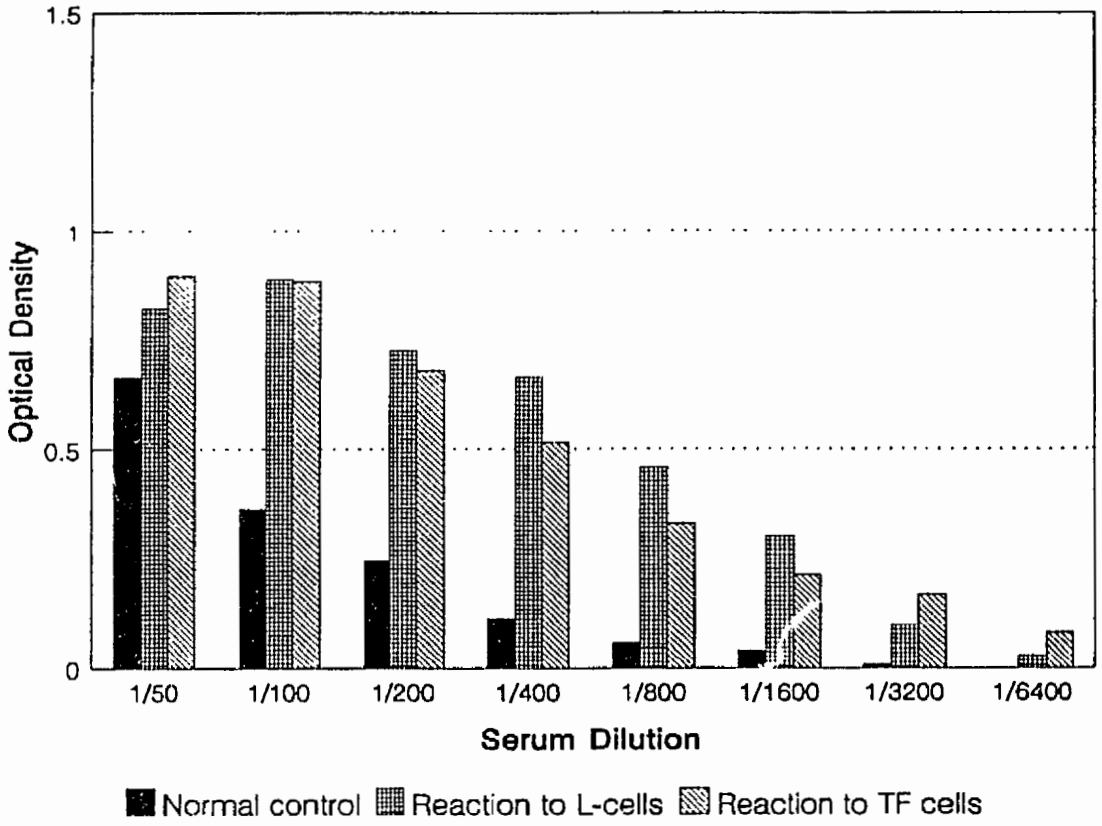

Figure 17. Serum antibody specificity in Balb/c mice

The antibody responses to L-cells and to L-cell DP transfectant cells were compared in Balb/c mice. The reactivitiy to L-cells and to DP cannot be distinguished from each other.

Usually $1x10^8$ spleen cells, or more are required for each fusion. If one could use a selected subset of spleen cells (such as antigen-specific B lymphocytes or IgG-bearing B lymphocytes) for fusion to partner cells, the labour of fusion, growth and screening of hybridomas would be greatly reduced. Meanwhile, the proportion of positive hybridomas should also be increased. Since, for most subsets, the number of selected spleen cells is too small for a standard PEG fusion, the centrifugal-electrofusion (CEF) method is preferable to the PEG fusion method.

The experiments in this section are based on an immunomagnetic fractionation principle using either antigen-coupled metallic beads or else metallic beads coupled to anti-mouse IgG. As the separation of IgG bearing B lymphocytes is easier technically than the separation of antigen-specific B lymphocytes, centrifugal-electrofusion of the IgG bearing spleen cells was the first method to be tried.

C.1. CEF of cells selected for surface expression of IgG

In this group of experiments, mouse spleen cells were collected and incubated with metallic beads coated with goat anti-mouse IgG (all subclasses) at 4°C for one hour. Then the cells bound to beads were separated using a magnet. The

selected spleen cells, still mixed with beads, were fused with SP2/o myeloma cells using centrifugal-electrofusion. After fusion, the cells were plated in 96-well culture plates with flat bottomed wells. Appropriate numbers of remainder cells (i.e. those remaining after the bead selection is completed) and unsorted spleen cells from the same spleen cell suspension were fused for comparison. Thus for each experiment, there were three fusion groups, 1) the selected cells; 2) the remainder cells; 3) the unsorted spleen cells.

a. Optimizing the conditions for hybridoma growth

Sixteen centrifugal electrofusions of cells selected for IgG expression were performed in an attempt to obtain hybridomas. Different conditions were tested in some experiments. The results are summarized in Table 1. Details of individual experiment are given in the appendix.

In fusions 1-2, the ratio of anti-mouse IgG metallic beads to spleen cells was 3:1 and 2:1 respectively. After fusion, when the cells were plated in 96-well culture plates, it was found that there were so many beads in each well that the cells could not be seen. No hybrids grew in any of the wells.

In fusions 3-5, the ratio of metallic beads to spleen cells was reduced to 1:2. The fused cells could now be observed in the 96 well plate. Some hybridoma colonies

Table 1. Summary of the centrifugal electrofusion trials using spleen cells selected with IgG-coated metallic beads.

Fusion Number	Mice	Ratio of Beads per Spleen Cell	Selected Cells from Spleen Population:		Feeder Cell Layer	Results	Note
			Number	Percent			
#1	C3H	3:1	$1.0x10^7$	5%	m RBC	no growth	bead layer too thick.
#2	C3H	2:1	$3.6x10^6$	2%	m RBC	signs of hybrids, but no growth	bead layer too thick.
#3	C3H	1:2	$2.5x10^7$	17%	m RBC	no growth	
#4	C3H C3H	1:2	$5.3x10^6$ $1.5x10^7$	1.6% 7.0%	m PMC	signs of hybrids, but no growth	
#5	Balb/c	1:2	$4.4x10^7$	20%	m RBC	some hybrids grew, then died	only in remainder & unsorted cell groups
#6	Balb/c	1:2	$1.9x10^7$	7%	m RBC	no growth	voltage was not appropriate
#7	Balb/c	1:2	$1.0x10^7$	7%	m PMC	some grew, but contaminated	influence of sucrose was tested

Table 1. (continued...)

Fusion Number	Mice	Ratio of Beads per Spleen Cell	Selected Cells from Spleen Population:		Feeder Cell Layer	Results	Note
			Number	Percent			
#8	Balb/c	1:2	2.8×10^7	23%	m PMC	no growth	influence of sucrose was tested
#9	Balb/c	1:2	1.5×10^7	11%	m RBC m PMC	no growth	pre-incubation was tested
#10	Balb/c	1:2	7.0×10^6	4%	m RBC	sign of hybrids, but no growth	taken care of by other person
#11	Balb/c	1:3	5.0×10^6	2%	m RBC	hybrids grew	reduced HAT concentration
#12	Balb/c	1:5	9.3×10^6	5%	m RBC	hybrids grew	reduced HAT concentration
#13	Balb/c	1:5	3.5×10^6	1.4%	m RBC	hybrids grew	reduced HAT concentration
#14	Balb/c	1:3	7.8×10^6	5%	m RBC	hybrids grew	reduced HAT concentration
#15	Balb/c	1:3	1.4×10^7	5.6%	m RBC	hybrids grew	reduced HAT concentration
#16	Balb/c	1:3	2.2×10^7	11%	m RBC	hybrids grew	reduced HAT concentration

appeared by day 7-10 after fusion in fusion 5 but they died 1-2 days later.

In fusion 6, the voltage for fusion was not appropriate due to equipment failure. There was no growth of hybridomas.

In fusions 7-10, attempts to improve the conditions for hybridoma growth were made. In fusion 7 and 8, after the centrifugal-electrofusion, half of the fused cells were washed twice with culture medium to remove any excess of the low ionic strength fusion buffer; the other half were plated directly without washing. In fusion 9, half of the fused cells were incubated at 37°C for 45 min, then plated; the other half were directly plated without incubation. In fusion 10, the possible influence of technical skill was tested by leaving the fused cells in the care of an experienced technician (instead of this graduate student). All these attempts failed to show any differences between two test groups.

Peritoneal macrophage cells were used as feeder cells in fusion 4, 7, 8 and 9; they did not appear to lead to any improvement in cell growth.

In fusions 11-16, attention was shifted onto the HAT medium used for feeding fused cells as a possible cause for poor cell survival. The modification introduced in fusion 11-16 was to reduce the concentration of HAT to half of the conventional concentration. A plate of SP2/o myeloma cells was set up as a control, to show that this HAT concentration was

still 100% inhibitory to them. The SP2/o cells were fed with the same HAT medium at the same time as the fused cells were fed. When all of the SP2/o cells died in the control plate, the HAT medium was changed to HT medium. In these experiments, the ratio of metallic beads to spleen cells was reduced further to 1:3 and 1:5. With these modifications, fusions 11-16 began to grow hybridomas. The growth and screening of these hybridomas are summarized in Table 2 and discussed in the following section.

b. Screening of the hybridomas

In fusions 11-16, the spleen cells were all from mice immunized with metallic beads which were supposedly loaded with DP2.1 molecules; the cell lysate had been made from the B cell line, WT51. The specificity of the hybridomas that grew was examined using the CELISA method.

The first screening, using WT51 B cell line cells (the immunogen), was considered a general selection. Positive hybridomas were counted and selected for further screening.

In the second screening, transfectant cells expressing different DP, DQ or DR molecules were employed as targets. This screen differentiated various antibodies with monomorphic specificity (anti class II HLA, anti all DP, anti DP and DR), and also revealed certain antibodies which reacted with polymorphic determinants on a number of DP molecules. However,

it should be mentioned here that the selection of specific hybridomas with possible polymorphic specificity was based on a relatively low optical density (OD) value as a cut off (>0.15).

In the third screening, the hybridomas selected from the second screen as being specific for DP transfectants were examined using a panel of homozygous B lymphoblastoid cell lines.

The results of fusions 11-16 and the outcomes of specificity screening are presented in Table 2. We can see, from the table, that fusions 12 and 15 were generally unsuccessful and were terminated at the end of the second week after fusion without any screening. In fusions 14 and 16, the first screen did not show the expected results; those too were terminated. Fusions 11 and 13 appeared productive. Many hybridomas were positive, particularly in the first screening. From these data the two ratios required for columns 7 and 8 of the table could be calculated; these are 1) the number of hybrids positive in the second screening per million cells fused and 2) the frequency of immunogen specific hybrids in relation to the total number of hybrids.

It is of interest to note that in both experiments where a reasonable number of hybrids was produced (fusions 11 and 13), a higher proportion of immunogen-specific cells was found with the metallic bead selected spleen cells. Thus the

Table 2. Growth and screening results of the hybridomas

Fusion Number	Number of Fused spleen cells	Total Number of hybrids formed	No. of +ve hybrids from Scrn 1 (immunogen)	No. of hybrids spec. for class II (Tfs, scrn 2)	No. of +ve hybrids from Scrn 3 (B-cell lines)	Ratio of hybrids spec. for class II over total # cells fused	Percent of hybrids spec. for class II over total # hybrids
#11	Sel. $5.2x10^6$	64	61	15	3	$2.9/10^6$	23%
	Rem. $1x10^7$	87	3	1	1	$0.1/10^6$	1.1%
	Uns. $1x10^7$	73	4	1	0	$0.1/10^6$	1.3%
#12	Sel. $9.3x10^6$	0					
	Rem. $9.3x10^6$	5	not tested				
	Uns. $9.3x10^6$	0					
#13	sel. $3.5x10^6$	57	13	7	1	$2/10^6$	12%
	Rem. $7x10^6$	87	11	6	2	$0.85/10^6$	6.8%
	Uns. $7x10^6$	89	9	5	1	$0.7/10^6$	5.6%

Table 2. (continued...)

Fusion Number	Number of Fused spleen cells	Total Number of hybrids formed	No. of +ve hybrids from Scrn 1 (immunogen)	No. of hybrids spec. for class II (Tfs, scrn 2)	No. of +ve hybrids from Scrn 3 (B-cell lines)	Ratio of hybrids spec. for class II over total # cells fused	Percent of hybrids spec. for class II over total # hybrids
#14	Sel. $7.8x10^6$	9	-ve				
	Rem. $7.8x10^6$	11	-ve				
	Uns. $7.8x10^6$	11	-ve				
#15	Sel. $1.4x10^7$	0					
	Rem. $1.4x10^7$	0					
	Uns. $1.4x10^7$	5	not tested				
#16	Sel. $2.2x10^7$	7	-ve				
	Rem. $2.2x10^7$	1	-ve				
	Uns. $2.2x10^7$	66	8	not tested			

selective procedure, although only for IgG positive cells, appeared to enrich for the cells of particular interest.

The hybridomas selected after the third screen were given to the immunology lab and the antibodies were tested against human peripheral blood B lymphocytes, using flow cytometry. Surprisingly, the supernatants did not show much reaction with B lymphocytes derived from blood samples. The same situation has been found in some other experiments in this lab; the discrepancy is not yet explained.

Conclusions from this series of experiments are twofold. First, they suggest that the metallic beads have picked up immunogenic DP molecules; second they show, in a preliminary fashion, that sorting IgG positive cells may, in a secondary response, be a useful way to select out immunogen reactive B cells for fusion.

C.2. Fusion of spleen cells fractionated with antigen coupled metallic beads

Several attempts to fuse spleen cells that had been selected using antigen coupled metallic beads were made. Metallic beads were coupled to DP molecules through physical absorption using a cell lysate made from WT51 B lymphoblastoid cell line cells. The DP-loaded beads were incubated with

immune spleen cell suspensions for one hour at 4°C. The spleen cells bound to beads were separated and fused to SP2/o myeloma cells using the centrifugal electrofusion technique. Three fusions were performed, which are summarized in Table 3. Fusion 17 did not produce many hybrids in the selected cell group; screening using WT51 B cell line cells as the target showed negative results. Fusion 18 only grew several hybridomas in control groups. The culture was terminated after two weeks. In fusion 19, hybridomas were produced in all three groups; unfortunately, the cultures were contaminated after preliminary testing had shown them to be positive for the immunizing cell; they were all lost.

The conclusion to these antigen-specific experiments is that, when all components of the technology are working satisfactorily, it appears that significant enrichment for antigen specific hybridomas is possible.

Table 3. Fusions of the selected cells fractionated by antigen coupled metallic beads

Fusion Number	Mice	Immunogen	Ratio of beads to Cell	Number of fused cells	Percent of spleen cell population	Number of hybrids	Number (and %) of +ve hybrids to immunogen
#17	Balb/c	Metallic beads loaded with DP molecules from WT51 B-cell line	1:5	Sel. $3x10^6$	1.5%	3	-ve
				Rem. $6x10^6$		34	-ve
				Uns. $6x10^6$		16	-ve
#18	Balb/c	Metallic beads loaded with DP molecules from WT51 B-cell line	1:5	Sel. $2.5x10^6$	1.6%	0	
				Rem. $5x10^6$		4	not tested
				Uns. $5x10^6$		2	not tested
#19	Balb/c	Metallic beads loaded with DP molecules from WT51 B-cell line	1:5	Sel. $3.7x10^6$	1.8%	17	9 (53%)
				Rem. $7x10^6$		26	2 (8%)
				Uns. $7x10^6$		34	5 (15%)

Chapter 6

DISCUSSION and CONCLUSIONS

In this study the investigation of using immunomagnetism in hybridoma production was done in three general parts: 1) antigen preparation, 2) immunization, 3) electrofusion of selected spleen cells. The discussion will be focused on the results of the experiments in these three parts.

A. SELECTION OF APPROPRIATE ANTIBODIES

At the beginning of this study, the preliminary work involved (i) choosing a capture antibody for immune absorption of HLA-DP molecules and (ii) developing an antibody-based indicator system. As both antibodies had to bind to the same molecule, it was imperative that they should not block each other.

A commercially available fluorescent antibody, I3, was chosen as an indicator to demonstrate HLA molecules absorbed onto metallic beads. This choice was based on an antigen specificity analysis which showed that I3 bound only DP expressing transfectant cells. However, as it was only

possible to test a limited range of antigens in the analysis, any proof that HLA-DP molecules were present, using I3 could only be partial; complementary demonstrations would be required.

The capture antibody was chosen through an experiment to test for cross blocking of antibody I3 (Drover et al. 1991). Results of the experiment provided two favourable conditions for the subsequent study of absorbed molecules. First, M67 specific for a monomorphic determinant on HLA-DP was shown not to block I3. This made M67 an ideal capture antibody that could be immobilized on metallic beads. Second, M58 was found to be a potent blocker of I3. This property provided a useful tool to confirm the presence of absorbed DP molecules. M58 is specific for an HLA-DP polymorphism, if a known interaction between the fluorescent antibody I3 and its target molecules could be blocked by M58, the target molecules would by inference be HLA-DP.

From the cross blocking tests, it can also be concluded that M67 and M58 will not block each other since they were shown in the experiment to bind to different locations of the same DP molecule. This property was used later for a direct demonstration of the presence of absorbed DP molecules on beads, in which M67 was labelled with biotin-avidin-FITC, while M58 acted as a capture antibody.

B. ABSORPTION OF DP MOLECULES

DP absorption from cell lysates onto metallic beads was done in two serial extraction experiments. In the first one, a standard number of beads was shown to have a gradual increase of absorbed DP molecules as the cell number in the cell lysates was increased. In the second one, serial absorptions were performed using a lysate made from a standard number of cells. When a large number of metallic beads was used, a low absorption density was noted, as compared with the use of a small number of beads, indicating a relative shortage of target molecules in the cell lysate. Although the two experiments can not be counted as quantitative, an approximate ratio for an effective use of metallic beads and cells in immune absorption of DP molecules can be inferred from the data. Using a B cell line for preparation of the lysate, reasonable saturation of the beads was shown when I used $1x10^6$ cells in the lysate and added about $2x10^6$ beads.

The measurement of DP molecules on cell membranes or in cell lysates, on a weight basis, was not done in this study. Such a determination would be quite difficult because the DP expression is considered to be at a low level. An approach to quantify DP molecules could probably be made by the serial extraction method. It was shown that with the use of a large number of metallic beads for the absorption, the DP molecules

in a cell lysate could be completely absorbed. If total protein eluted from DP absorbed beads and total protein from a matching lot of unabsorbed beads could be determined, the amount of DP molecules could be calculated by subtraction. The problem is that in both cases there would be immunoglobulin released which would give a significant background reading.

The specific absorption of DP molecules by metallic beads was shown by three experiments. In the first one, the metallic beads were coated with M67; the absorbed molecules were identified by FITC-labelled antibody I3. It was noted that the fluorescent antibody I3 only stained the beads that had been absorbed in a cell lysate containing DP molecules; it did not stain beads that had been absorbed in a lysate lacking DP molecules, nor when the beads were coated with an irrelevant antibody. This provides good evidence that the anti-DP antibody coated metallic beads made a specific absorption from a B cell line cell lysate.

In the second experiment, the specific absorption was confirmed by the M58 blocking test, in which the metallic beads with absorbed molecules were first incubated with M58, then stained by fluorescent antibody I3. As mentioned before, M58 is specific for a DP polymorphism; thus if the binding of the fluorescent antibody I3 and the absorbed molecules is blocked by M58, this is highly suggestive that the absorbed molecules on metallic beads should be DP.

The third experiment provided direct evidence that the metallic beads absorb DP specifically. In this experiment, M58 as a capture antibody was coated onto metallic beads, while M67 was conjugated with biotin, serving as an indicator. As both antibodies are DP specific, there is no doubt that the molecules identified on metallic beads are DP molecules.

It was supposed that some non-specific binding of DQ and DR class II molecules or other non-MHC antigens might occur in "DP specific absorptions" as a result of some portions of cell membrane with embedded membrane molecules being incompletely reduced in the lysate and present in the form of micelles. Thus, all kinds of membrane proteins would likely be picked up by the beads. This problem was prevented in the DP absorption experiment by an immediate washing of metallic beads with fresh lysing buffer after the absorption had been completed.

C. IMMUNIZATION

The use of metallic beads absorbed with HLA-DP molecules for immunization was prompted by two advantages. Firstly, the immunogenicity of small doses of such molecules is increased by this procedure (Goding 1986, Nilsson et al. 1990). Secondly, the preparation of antigen by this method is

less time-consuming since the procedure of elution and dialysis of the purified molecules is eliminated. Antisera from the immunized mice were tested by assessing their reaction with the cells that had been used for preparation of antigens. The antibody response was seen clearly. Ideally, the specificity of antibodies in these antisera should be tested to determine how much of the reaction was against HLA-DP and how much to other B cell molecules including HLA-DR and -DQ. However, as much of the response would be against monomorphic determinants on these molecules, it would be very difficult to quantify the response among these molecules. For this and other reasons it was decided not to attempt such an analysis.

In a comparison between the use of beryllium sulphate and complete Freund's adjuvant, a significant primary antibody response was not shown. However, there was a clear difference in antibody titre between two groups in the secondary antibody response, although in the secondary immunization no beryllium sulphate or CFA was used. As the difference between the two groups of mice is consistent, we attribute it to the influence of the two adjuvants used in the primary immunization. Freund's adjuvant gave the better result.

It is known that the beryllium sulphate is a carcinogenic chemical compound. This was revealed in the present study. Three out of the eight mice that received beryllium sulphate as an adjuvant developed tumours later at

the site of injection. The malignancy was confirmed by histopathological study of the tumours. This is a further reason for preferring Freund's complete adjuvant.

L cells, which are of C3H origin, have been found to cause a weak antibody response in C3H mice, whether in this laboratory or in this study. The reason for this phenomenon is uncertain. However, since the L cells are mutants of tumour type of fibroblasts, there could possibly be some variances in the composition of the cell membrane molecules.

D. PRE-FUSION CELL SELECTION

The use of metallic beads for pre-fusion cell selection of specific spleen cells is a relatively unexplored approach to hybridoma creation. So far, the only reported study of this approach has been that of Egeland et al. (1988), in which rabbit IgG coupled metallic beads were used to rosette rheumatoid factor positive B lymphocytes. The rosetted cells, after having been transformed with Epstein-Barr virus for bulk expansion, were fused with myeloma cells. The usefulness of Egeland's method is limited because cells other than human B lymphocytes cannot be transformed with EB virus for amplification, while on the other hand the routine method of PEG fusion is only applicable to large numbers of cells. The

centrifugo-electrofusion facility devised by the immunology laboratory of Memorial University of Newfoundland provided an excellent tool for the fusion of a small number of cells and made the exploration of specific fusion of selected mouse spleen cells possible. In this study, pre-fusion cell selection was investigated in two groups of experiments. One was with the use of goat anti-mouse IgG (GAM) coupled metallic beads, the other one was with the use of the specific antigen, HLA-DP molecules, coupled to metallic beads.

D.1. Selection of IgG bearing cells

GAM-IgG coupled metallic beads were used in an attempt to rosette IgG expressing lymphocytes in spleen cell suspensions. Previous reports suggested that the ratio of metallic beads to cells for positive cell selection should be 3 to 1. However, we found in this study that such a ratio caused a thick layer of beads to form on the bottom of cell culture wells, which completely obscured observation of the fused cells. A ratio of one bead to three or five cells was adopted in later experiments. Various yields of selected cells were shown in cell fractionations, but no consistent tendency could be seen in these fluctuating data. This makes a comment on the efficiency of the metallic beads in the cell

selections difficult. To solve this problem, a specific analysis of the number of cells expressing IgG molecules in spleen cell suspensions would be required before and after cell selection, as well as checking the purity of the selected cells. Flow cytometry would be good for this purpose.

A relatively higher percentage of positive hybridomas was achieved in the fusions of the selected spleen cells, suggesting that the positive hybridoma frequency could be increased by specific fusion of the IgG expressing lymphocytes. This is possible since, as the fusions were performed on the third day after boost immunization, most antigen-specific lymphocytes should be among the population of cells expressing IgG.

To evaluate the selection of IgG bearing cells, an antibody isotyping test would be interesting. Theoretically, most antibodies from the fusion of the cells selected by GAM-IgG beads are IgG isotype, while those from the fusion of the remainder cells are mostly IgM isotype. Future experiments of this nature should include isotyping.

D.2. Selection of antigen-specific lymphocytes

Selection of DP-specific B lymphocytes was made by using HLA-DP coupled metallic beads. This should be more

difficult than the selection of IgG bearing lymphocytes since the number of the target cells in a spleen cell suspension is much smaller. The data from the experiments in this study are fragmentary. However, in one of the fusions of the cells selected with DP antigen coupled beads, a higher percentage of positive hybridomas was produced than in the control fusions. This is a hopeful finding to encourage further explorations.

E. GROWING ELECTROFUSED HYBRIDOMAS

The growth of hybridoma cells in this study went through an initial period of difficulty. In the first ten fusions the hybridomas had died one to two weeks after the fusion for no detectable reason. Two factors were considered to be the cause of this phenomenon. Mycoplasma contamination is assumed to be one important factor, as at a later stage of this study, mycoplasma was identified in several cell lines in this lab, including the fusion partner line, SP2/o. Mycoplasma has been commonly found to be the major agent in cell culture contamination in many laboratories. Mycoplasma infection has been shown to affect the ability of cells to form hybridomas (Goding 1986). However, the way in which mycoplasma infection causes cell death is not certain. It is also not known why such contamination causes hybridoma death, but not death of

established cell lines.

A second factor that was considered in the search for the cause of hybridoma death was the concentration of HAT in the cell culture medium. Hybridomas finally grew in a medium with a reduced concentration of HAT, suggesting that the concentration of the HAT might play a role in the failure of the hybridoma growth. Considering both factors together, It is not impossible that mycoplasma contamination not only interferes with hybridoma growth but also causes the hybridoma to be excessively vulnerable to the toxicity of HAT medium. It would be rewarding to do a series of experiments in which electrofused cells are separately grown in several kinds of medium with different concentrations of HAT.

Malfunction of the electrical apparatus has been an occasional cause of failure in fusion experiments in this lab. Other studies on electrofusion in the same lab have shown that the voltage, pulse duration and cell number all affect hybridoma formation. However, up to now no evidence has been found that electrical factors could cause formed hybridomas to die.

A slightly different way to plate fused cells was used in some of the fusion experiments in this project. It was noted that in several fusions, due to the small number of selected spleen cells, there were only enough cells to plate a few culture wells when the standard number of cells per well

($1.5x10^5$) was adhered to. The number of metallic beads in each well was relatively increased and this may have inhibited hybrid growth and also prevented detection of the hybrids . The fused cells were finally plated in more wells than the usual, with slightly less than $1x10^5$ cells per well. In order to avert the negative effect of small number of cells in each well on hybrid growth, sufficient non-fused spleen cells were added so that the number of $1.5x10^5$ cells was kept in each well. The successful growth of hybrids in these plates confirmed this plating method's feasibility.

F. SCREENING HYBRIDOMAS

Study of the specificities of some of the monoclonal antibodies that were produced from these experiments is important in so far as these reflect on the effectiveness of the methods employed. In particular the finding of anti-DP antibodies supports the idea that immunisation with DP absorbed onto beads was effective. In addition, the proof of the value of the cell selection experiments (pre-fusion selection) lies in the yield of hybridomas with anti-DP specificity. The specificities found at each level of screening are now discussed.

The hybridomas were examined in three screenings. The

first was a general selection in which all hybridomas reacting with the cells that had been used for preparation of immunogens were selected. Although DP specificity cannot be distinguished in this screening, the higher percentage of positive hybridomas produced by fusion of selected spleen cells is helpful in assessing the effect of pre-fusion selection of immune spleen cells.

The second and third screenings were specificity assays. In the second, DQ, DR and DP transfectant cells were used as targets. Identification of potentially useful hybridomas at this stage is based on an overall balance of the optical density recorded and on the preliminary determination of antibody specificity. Some hybridomas having a high OD value in the assay but not showing specificity for a DP polymorphism were not selected for further study. Only those specific for DP transfectants were picked. It was noted that, sometimes, the antibodies showing weak affinity for transfectant cells had a good reactivity with B cell lines. The reason for this is not known. Since the expression of HLA-DP on cell lines is more natural than it is on transfectants, further specificity information was obtained by studying the antibody's reactivity to B cell lines.

Hybridomas showing specificity for either DP polymorphisms or class II monomorphisms were counted in each successful fusion. This hybridoma frequency was found to be

higher in the fusion of selected spleen cells than in the control fusions of remainder or of unsorted spleen cells. This result confirmed the feasibility of the pre-fusion selection method in hybridoma production. Moreover, the DP specificity of these hybridomas provided further direct evidence that DP molecules absorbed onto metallic beads can be used to provoke an antibody response in mice.

G. CONCLUSIONS

Metallic beads were used at many points in the course of monoclonal antibody production, from antigen preparation to hybridoma formation. The advantage of using metallic beads in each procedure is evident. As the metallic beads can be manipulated with a magnet, all the procedures that involve their use become convenient and time saving. Metallic beads coated with specific antibody are effective in immune absorption of HLA-DP molecules from cell lysates. This was confirmed by the demonstration of DP molecules using the fluorescent antibody I3, and by use of a combination of the antibody M58 and biotinylated MG7. Metallic beads carrying absorbed DP molecules are immunogenic. This was shown in the antiserum testing. With the direct use of DP molecules absorbed onto metallic beads, the cumbersome procedures of

elution and dialysis of purified antigen molecules were eliminated. The use of metallic beads for pre-fusion cell selection is feasible. Although there is no available data to show directly the efficacy of these beads on selection of IgG-expressing or antigen-specific lymphocytes in a spleen cell suspension, the higher frequency of positive hybridomas produced in this project, in the fusions of the selected spleen cells, indicates that the application of these beads to pre-fusion cell selection is a possible way to improve the frequency of antigen-specific hybridomas.

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APPENDIX

Fusion 1. Mouse: C3H

24/07/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from DP-expressing transfectant cells (DPB1*0901) in primary, secondary immunization, as well as in pre-fusion boost.

Spleen cells: $1.9x10^8$

Cell selection: About $6x10^8$ metallic beads coated with GAM-IgG antibodies were mixed with spleen cells. After an incubation at 4°C for half an hour, about $1x10^7$ cells were pulled out (only rosettes were counted).

SP2/o cells: $4x10^6$

Fusion conditions: Half the selected cells were fused with SP2/o cells covering on the bottom of fusion chamber (2 pulses, 2.25kV/cm and 100μSec in each one); another half cells were fused in a cell mixture manner (2 pulses, 2.25kV/cm and 100μSec in each one).

Feeder cells: Normal mouse red blood cells

Plated cells: In 2x70 wells, $1x10^5$ cells per well.

Results: No growth; the layer of metallic beads

looked thick. Control fusions of remainder cells and unsorted spleen cells were not done.

Fusion 2. Mouse: C3H

26/07/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from DP-expressing transfectant cells (DPB1*0901) in primary and secondary immunization. $2x10^7$ transfectant cells of the same nature in pre-fusion boost.

Spleen cells: $1.8x10^8$

Cell selection: About $3.6x10^8$ metallic beads coated with GAM-IgG antibodies were mixed with those spleen cells. After an incubation at 4°C for half an hour, about $3.6x10^6$ cells were pulled out (only rosettes were counted).

SP2/o cells: $1.8x10^6$

Fusion conditions: All the cells were fused with SP2/o cells covering on the bottom of fusion chamber. Two different pulses were delivered, one was 2.23kV/cm, 100μSec; the other one was 2.23kV/cm, 200μSec.

Feeder cells: Normal mouse red blood cells

Plated cells: In 50 wells, $1x10^5$ cells per well. Half the fused cells were put in a magnetic field after fusion, in an attempt to separate the cells

from the metallic beads. Another half cells were plated directly, without the magnetic separation. Results: No growth. The layer of metallic beads looked thick. Control fusions of remainder cells and unsorted spleen cells were not done.

Fusion 3. Mouse: C3H

05/08/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a lysate made from DP-expressing transfectant cells (DPB1*0401) in primary and secondary immunization, as well as in pre-fusion boost.

Spleen cells: $1.4x10^8$

Cell selection: About $7x10^7$ metallic beads coated with GAM-IgG antibodies were mixed with those spleen cells. After an incubation at 4°C for one hour, a total of $2.5x10^7$ cells were pulled out.

SP2/o cells: $5x10^6$

Fusion conditions: The fusion was done in a cell mixture manner, with 2 pulses, 2.25kV/cm and 100μSec in each one.

Feeder cells: Normal mouse red blood cells.

Plated cell: $1x10^5$ per well. Half the fused cells were incubated in a flask overnight. 10ml of an irrelevant antibody supernatant (M2, IgG isotype) was added into the flask in an attempt to detach the

beads from the cells. Then they were plated.

Results: No growth. Control fusions of unsorted cells and remainder cells were not done.

Fusion 4. Mouse: C3H (2 mice)

09/08/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a lysate made from DP-expressing transfectant cells (DPB1*0901, or DPB1*0401) in primary and secondary immunization, as well as in pre-fusion boost.

Spleen cells: $3.4x10^8$ (mouse one); $2.2x10^8$ (mouse two)

Cell selection: Half of the $3.4x10^8$ spleen cells (mouse one) were mixed with about $8x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $5.3x10^6$ cells were pulled out. Half of $2.2x10^8$ spleen cells (mouse two) were mixed with about $5x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $1.5x10^7$ cells were pulled out.

SP2/o cells: (1) $1.3x10^6$ to $5.3x10^6$ selected spleen cells; (2) $3.8x10^6$ to $1.5x10^7$ selected spleen cells.

Fusion conditions: Two fusions were done separately in a cell mixture manner. In each fusion, two

pulses, 2.25kV/cm and 100μSec in each pulse were delivered.

Feeder cells: Mouse peritoneal phagocytes.

Plated cells: In 72 wells, about $1x10^5$ cells per well.

Results: Several wells had signs of hybrids, but there was no further growth.

Fusion 5. Mouse: Balb/c

05/09/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from DP-expressing transfectant cells (DPB1*0401) in primary and secondary immunization, as well as in pre-fusion boost.

Spleen cells: $2.2x10^8$

Cell selection: $1.7x10^8$ of those cells were mixed with $8.5x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for two hours, a total of $4.4x10^7$ cells were pulled out.

SP2/o cells: $8.8x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.28kV/cm and 100μSec in each one, were delivered.

Feeder cells: Normal mouse red blood cells.

Plated cells: In 352 wells, $1.5x10^5$ cells per well.

Results: In control groups (fusions of unsorted spleen cells from the same mouse and remainder cells), some hybrids grew for a couple of days, then died.

Fusion 6. Mouse: Balb/c

18/09/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $2.5x10^8$

Cell selection: $1.6x10^8$ of those spleen cells were mixed with $8x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for 1.5 hour, a total of $1.9x10^7$ cells were pulled out.

SP2/o cells: $4x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.17kV/cm and 100μSec in each one, were delivered.

Feeder cells: Fresh mouse red blood cells

Plated cells: In 152 wells, about $1.5x10^5$ cells per well.

Results: Fusion facilities did not work properly. There was no growth.

Fusion 7: Mouse: Balb/c

25/09/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $2x10^8$

Cell selection: $1.5x10^8$ of those spleen cells were mixed with $7.5x10^7$ metallic beads. After an incubation at 4°C for one hour, a total of $1.1x10^7$ cells were pulled out.

SP2/0 cells: $2.5x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.19kV/cm and 100μSec in each one, were delivered.

Feeder cells: Mouse peritoneal phagocytes

Plated cells: In 96 wells, about $1.5x10^5$ cells per well. Half the fused cells were washed twice with culture medium, then plated. Another half cells were directly plated without washing.

Results: In control groups (fusions of unsorted spleen cells from the same spleen and remainder cells), some hybrids grew for a couple of days, also culture was contaminated.

Fusion 8. Mouse: Balb/c

03/10/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $1.6x10^8$

Cell selection: $1.2x10^8$ of those spleen cells were mixed with $6x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $2.8x10^7$ cells were pulled out.

SP2/o cells: $5.6x10^6$

Fusion conditions: Since the number of cells was above the upper limit for one fusion, the cells were divided and each of the two halves was fused with SP2/o cells in a cell mixture manner. Two pulses were delivered to each fusion; 2.18kV/cm and 100μSec in the first fusion, 2.14kV/cm and 100μSec in the second fusion.

Feeder cells: Mouse peritoneal phagocytes

Plated cells: Half the fused cells were washed with culture medium, then they were plated with about $1.5x10^5$ per well; another half cells were plated directly without washing, with about $1.5x10^5$ per well.

Results: No growth

Fusion 9. Mouse: Balb/c

08/10/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $1.7x10^8$

Cell selection: $1.3x10^8$ of those spleen cells were mixed with $6x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $1.5x10^7$ cells were pulled out.

SP2/o cells: $3x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses were delivered, 2.27kV/cm and 100μSec in each one.

Feeder cells: Half the fused cells were fed with both mouse peritoneal phagocytes and red blood cells; another half cells were fed with mouse red blood cells only.

Plated cells: Half the fused cells were incubated at 37°C for 30min after fusion, then they were plated in 64 wells, about $1.5x10^5$ cells per well; another half cells, without incubation, were directly plated in 64 wells, about $1.5x10^5$ per well.

Results: No growth in any well.

Fusion 10 Mouse: Balb/c

18/10/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $1.5x10^8$

Cell Selection: About $1.2x10^8$ of those spleen cells were mixed with $6x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $7x10^6$ cells were pulled out.

SP2/o cells: $1.5x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.29kV/cm and 100μSec in each one, were delivered.

Feeder cells: Normal mouse red blood cells

Plated cells: In 56 wells, $1.5x10^5$ per well. After being plated, the cells were taken cared for by an experienced technician.

Results: Some signs of hybrids, but no further growth.

Fusion 11 Mouse: Balb/c

28/10/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells

in pre-fusion boost.

Spleen cells: $3.5x10^8$

Cell selection: About $2.4x10^8$ of those spleen cells were mixed with $8x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $5.2x10^6$ cells were pulled out.

SP2/o cells: $1x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.33kV/cm and 100μSec in each one, were delivered.

Feeder cells: Normal mouse red blood cells

Plated cells: The fused cells were plated in 64 wells, less than $1x10^5$ cells per well. In order to avert the negative effect of the low number of cells on hybrid growth, sufficient non-fused spleen cells were added in so that the number of $1.5x10^5$ cells were kept in each well. 1x HAT medium in the first feeding, and 1/2x HAT in the subsequent feeding were used. Meanwhile, a plate of SP2/o cells, as an indicator, were fed with the same HAT medium. When all these SP2/o cells were dead, The HAT medium was changed to HT medium.

Results: Many hybrids grew

Fusion 12 Mouse: Balb/c

07/11/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $2.3x10^8$

Cell selection: About $1.8x10^8$ of those spleen cells were mixed with about $3.6x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $9.3x10^6$ cells were pulled out.

SP2/o cells: $2x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.33kV/cm and 100μSec in each one, were delivered.

Feeder cells: Normal mouse red blood cells

Plated cells: About $1.5x10^5$ cells per well. 1x HAT medium was used in the first feeding.

Results: 5 wells in control fusion of remainder cells had hybrids growing. The culture was terminated.

Fusion 13 Mouse: Balb/c

14/11/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells

in pre-fusion boost.

Spleen cells: $2.98x10^8$

Cell selection: About $2.5x10^8$ of those spleen cells were mixed with $5x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $3.5x10^6$ cells were pulled out.

SP2/o cells: About $7x10^5$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.33kV/cm and 100μSec in each one, were delivered.

Feeder cells: Normal mouse red blood cells

Plated cells: The fused cells were plated in 80 wells, with less than $1x10^5$ cells per well. In order to avert the negative effect of lower number of cells on hybrid growth, sufficient non-fused spleen cells were added so that a number of $1.5x10^5$ cells was kept in each well. 1x HAT medium in the first feeding, and 1/2x HAT in the subsequent were used.

Results: Many hybrids grew.

Fusion 14 Mouse: Balb/c

18/11/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary; $2x10^7$ WT51 cells in secondary immunisation; 5x107 metallic beads loaded with the same molecules

in pre-fusion boost.

Spleen cells: $1.9x10^8$

Cell selection: $1.5x10^8$ of those spleen cells were mixed with $5x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $7.8x10^6$ cells were pulled out.

SP2/0 cells: $1.6x10^6$

Fusion conditions: Fusion was done in a cell mixture manner. Two pulses, 2.32kV/cm, 100μSec.

Feeder cells: Normal mouse red blood cells

Plated cells: In 96 wells, non-fused spleen cells were added so that each well had at least $1.5x10^5$ cells.

Results: Hybrids grew

Fusion 15 Mouse: Balb/c

24/01/92 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunisation; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $2.9x10^8$

Cell selection: $2.5x10^8$ of those spleen cells were mixed with $8x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $1.4x10^7$ cells were pulled out.

SP2/o cells: $3x10^6$

Fusion conditions: Cells in a mixture manner; two pulses, 182kV/cm, 100μSec.

Feeder cells: Normal mouse red blood cells

Plated cells: In 112 wells, $1.5x10^5$ cells per well.

Results: Hybrids grew

Fusion 16 Mouse: Balb/c

31/01/92 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary and secondary immunization; $2x10^7$ WT51 cells in pre-fusion boost.

Spleen cells: $2.3x10^8$

Cell selection: $2x10^8$ of those spleen cells were mixed with $6.5x10^7$ metallic beads coated with GAM-IgG antibodies. After an incubation at 4°C for one hour, a total of $2.2x10^7$ cells were pulled out.

SP2/o cells: $4.5x10^6$

Fusion conditions: Cells in a mixture manner; two pulses, 200kV/cm, 30μSec.

Feeder cells: Normal mouse red blood cells.

Plated cells: In 192 wells, about $1.4x10^5$ cells per well.

Results: Hybrids grew.

Fusion 17 Mouse: Balb/c

22/11/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary immunization; $2x10^7$ WT51 cells in secondary immunization and pre-fusion boost.

Spleen cells: $2.5x10^8$

Cell selection: $2x10^8$ of those spleen cells were mixed with $4x10^7$ metallic beads loaded with DP molecules. After an incubation at 4°C for one hour, a total of $3x10^6$ cells were pulled out.

SP2/0 cells: $6x10^5$

Fusion conditions: Cells in a mixture manner; two pulses, 2.32kV/cm, 100μSec.

Feeder cells: Normal mouse red blood cells

Plated cells: In 64 wells, non-fused spleen cells were added so that $1.5x10^5$ cells were kept in each well.

Results: Hybrids grew; negative in the first screening.

Fusion 18 Mouse: Balb/c

29/11/92 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary immunization; $2x10^7$ WT51 cells in secondary immunization; and $5x10^7$ metallic beads loaded with

the same DP molecules in pre-fusion boost.

Spleen cells: $1.7x10^8$

Cell selection: $1.5x10^8$ of those spleen cells were mixed with $3x10^7$ metallic beads loaded with DP molecules. After an incubation at 4°C for one hour, a total of $2.5x10^6$ cells were pulled out.

SP2/o cells: $5x10^5$

Fusion conditions: Cells in a mixture manner; two pulses, 2.31kV/cm, 100μSec.

Feeder cells: Normal mouse red blood cells

Plated cells: In 32 wells, non-fused spleen cells were added so that $1.5x10^5$ cells were kept in each well.

Results: Hybrids grew only in control fusions (unsorted spleen cells and remainder cells).

Fusion 19 Mouse: Balb/c

09/12/91 Antigen: $5x10^7$ metallic beads that had been absorbed in a cell lysate made from WT51 B cell line cells in primary immunization; $2x10^7$ WT51 cells in secondary immunization and pre-fusion boost.

Spleen cells: $2.7x10^8$

Cell selection: $2x10^8$ of those spleen cells were mixed with $4x10^7$ metallic beads loaded with DP molecules. After an incubation at 4°C for one hour,

a total of $3.7x10^6$ cells were pulled out.

SP2/o cells: About $7.5x10^5$

Fusion conditions: In a cell mixture manner; two pulses, 2.31kV/cm, 100μSec

Feeder cells: Normal mouse red blood cells

Plated cells: In 84 wells, non-fused spleen cells were added so that $1.5x10^5$ cells were kept in each well.

Results: Hybrids grew; positive in the first screening, then contaminated.