POST-FUSION SELECTION OF ANTIGEN-SPECIFIC HYBRIDOMAS

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By

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ABSTRACT

The ability to create monoclonal antibodies has allowed great strides to be made in research and clinical medicine, and continues to contribute to progression in many areas. However, the technology involved is labor-intensive and often inefficient. A typical cell hybridization procedure will generate thousands of hybridomas, with the great majority being irrelevant. Conventional technology requires maintenance and testing of the total population, in order to identify the very few hybrids that are secreting antibody of the desired specificity. Particularly for antigens of low immunogenicity, this is clearly inefficient.

The purpose of this study is to explore methods for selecting antigen-specific hybridomas soon after the fusion procedure. This would eliminate the unnecessary maintenance of irrelevant hybrids, reducing much of the time, effort and materials that are currently consumed in this technology.

For these fusions, transfectant cells expressing HLA-DP molecules were the immunogens. Monoclonal antibodies recognizing DP polymorphisms were desired as serologic reagents, for matching donor-recipient pairs in bone marrow transplantation. Fused cells were grown as bulk cultures and Ag-specific selection was attempted, using the antigen as a probe for hybridomas expressing specific immunoglobulin receptors. Two methods were tested: immunomagnetism (by use of antigen-coated immunometallic beads), and panning (by use of the immunizing transfectant cells). The yield of antigen-specific hybridomas was compared to that obtained in conventional fusions.

The results indicate that panning, using the procedure outlined in this study, is not a useful method for selecting DP-specific hybridomas from a post-fusion bulk population. Immunomagnetism, on the other hand, produced satisfactory results, offering a potentially useful way to increase the efficiency of monoclonal antibody generation.

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LIST OF ABBREVIATIONS

Ab	Antibody(ies)
ABC	Antigen-binding cell
Ag	Antigen(s)
BCL	B cell line
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CELISA	Cellular enzyme-linked immunosorbant assay
CFA	Complete Freund's adjuvant
CFDA	Carboxyfluorescein diacetate
CMV	Cytomegalovirus
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
EBV	Epstein Barr virus
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanin
GAM	Goat anti-mouse
HAT	Hypoxanthine/aminopterin/thymidine
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
I3	A mouse mAb specific for HLA-DP molecules
Ig	Immunoglobulin
IHW	International Histocompatibility Workshop

IP	Intraperitoneal
IV	Intravenous
mAb	Monoclonal antibody(ies)
MHC	Major histocompatibility complex
NFLD.M58	A mouse mAb specific for DPβ1:55-56:DE
NFLD.M67	A mouse mAb specific for HLA-DP molecules
NMS	Normal mouse serum
NNP	4-hydroxy-3,5-dinitrophenacetic acid
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PFC	Plaque-forming cell
PMSF	Phenylmethylsulfonyl fluoride
RAM	Rabbit anti-mouse
RBC	Red blood cell
RFC	Rosette-forming cell
RT	Room temperature
SC	Subcutaneous
sIg	Surface immunoglobulin
snf	Supernatant fluid
TNP	Trinitrophenyl

1.0 INTRODUCTION

The creation of hybridoma cells which secrete monoclonal antibodies (mAb), by somatic cell hybridization, was first described by Köhler and Milstein (1975, 1976). This technique allows the production of mAb *in vitro* by fusing spleen cells from immune mice with cells from a myeloma cell line. The resulting hybridomas can produce virtually unlimited quantities of homogeneous antibody. With improvements to the method (Gefter et al, 1977; Schulman et al, 1978; Fazekas de St. Groth et al, 1980; Westerwoudt, 1985), the basic approach of Köhler and Milstein has gained wide acceptance and become a valuable standard technique in many laboratories.

Compared to polyclonal antisera, which exhibit extreme heterogeneity, monoclonal antibodies are exquisitely specific. The ability to produce large quantities of mAb, with specificity for a desired antigenic determinant, has had far-reaching effects, not only on the field of immunology, but on many areas of research and clinical medicine. The diverse array of current applications for mAb include diagnosis of infectious and systemic diseases, tumor diagnosis and therapy, identification of phenotypic cell markers, and functional analysis of cell surface and secreted molecules.

Although mAb technology has proven to be highly successful, it is not without need for improvements. The main problem with the present method is that it is laborious and time-consuming

and, particularly for antigens of low immunogenicity, very inefficient. This means that, for many antigens (Ag), the frequency of Ag-specific hybridomas will be extremely low, requiring many non-productive fusion attempts. The reasons for this include the extremely low number of B cells that are specific for any given antigenic determinant, the low frequency of cell fusion, and the randomness of the fusion procedure. Taken together, the chance of isolating an Ag-specific hybridoma becomes very low indeed. As Köhler and Milstein once said "an antibody-producing hybrid line is a gift of the Gods".

Figure 1 gives a flow chart indicating the basic steps involved in the creation of hybridomas secreting mAb. Current methods rely on dispersion of fused cells in limiting dilution microculture for physical isolation of the hybrid clones produced. These cultures must be maintained and monitored and, when sufficiently expanded, each culture must be screened for specific antibody production. A look at the numbers involved gives some idea of the inefficiency of this technique.

A typical immunized mouse spleen will generate approximately 1-1.5 x 10^8 B cells. The frequency of cell fusion is estimated at about 10^{-5} (Casali et al, 1986). Therefore it follows that 1000-1500 hybridomas will be produced per fused spleen. Since the number of irrelevant B cells always far outnumbers the Ag-specific cells, very few (if any) of these fused cells will be

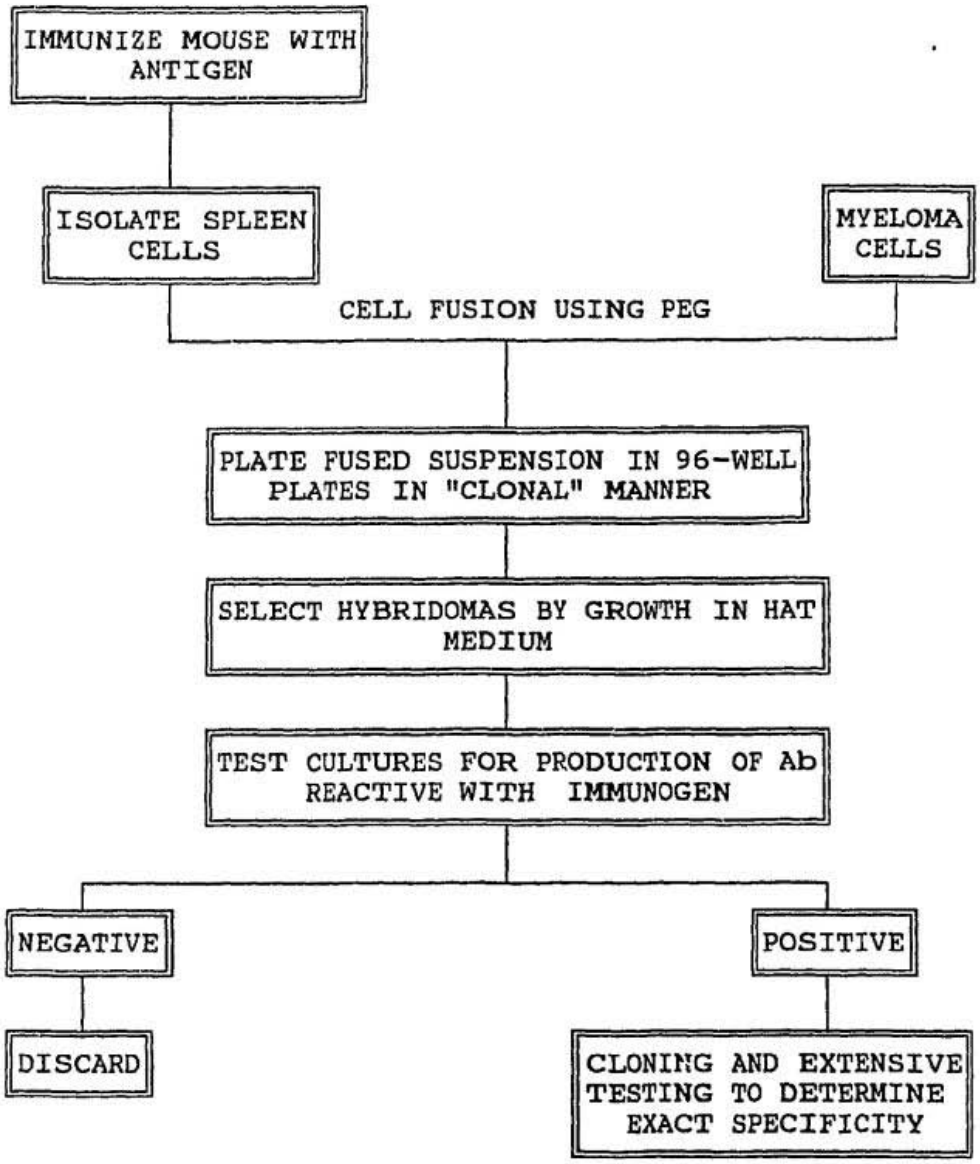


FIGURE 1. Diagram illustrating the basic steps involved in the identification and isolation of antigen-specific hybridomas using conventional, polyethylene-glycol mediated cell hybridization techniques.

secreting Ag-specific Ab.

The fused cells are plated in such a manner that approximately one hybrid can be expected per well; this means that ten to fifteen 96-well plates can be expected to result from a typical fusion. Maintaining and screening these 1000-1500 cultures to find the very few that contain the desired clones clearly is inefficient. Cultures must be fed every two to three days with selective medium, and growth of the hybridomas must be carefully monitored microscopically, as overgrowth can quickly result in death of the cells. When it is decided that the cultures have grown sufficiently, individual supernatants must be screened for specific antibody. Ag-specific hybrids must be identified and cloned as quickly as possible, as they may otherwise be lost due to overgrowth by unwanted hybrids or by nonproducing variants of the desired hybrid. Thus isolation of hybridoma lines with the desired reactivity becomes a painstaking procedure, impeding the generation of mAb to a wide range of Ag.

An ideal solution to this inefficiency would be Ag-specific selection, in which only the Ag-specific hybridomas are plated and screened. This type of selection is theoretically possible due to the fact that receptors (ie immunoglobulin (Ig) molecules) for Ag are expressed on the surfaces of B cells and hybridomas. Cells capable of making Ab with a desired specificity can, in principle, be separated from irrelevant

cells by using the Ag as a probe. Both pre-fusion selection of Ag-specific B cells and post-fusion selection of Ag-specific hybridomas are possible in principle.

Such a selection method would drastically decrease the number of hybridomas requiring maintenance and testing, while at the same time increase the yield of positive hybridomas. In theory, a typical fusion could be reduced from 1500 cultures to less than 100. Most (if not all) of these would be Ag-specific. This, of course, would reduce much of the time, effort and materials that are currently consumed in this technology. In addition, fusion of multiple mouse spleens would then be manageable, thereby increasing the probability of producing the mAb of interest.

The purpose of this thesis was to attempt Ag-specific selection shortly after the fusion procedure, in an effort to increase the efficiency of mAb production. Polyethylene glycol (PEG) is used as the fusogenic agent. Two selection methods are described, namely immunomagnetism and panning, and the yield of Ag-specific hybridomas is compared to that obtained using conventional PEG fusions, without selection.

Most attempts to improve the efficiency of cell fusion have concentrated on modifications to the existing procedure. Fewer attempts have been made to improve the selectivity of the method. In recent years, however, Ag-specific selection for the purpose of mAb production has been attempted, using

several different techniques. Some of the pertinent literature on this subject will now be reviewed.

1.1 REVIEW OF EARLY ATTEMPTS TO SELECT ANTIGEN SPECIFIC CELLS

Early attempts to isolate Ag-specific B cells were done, not for the purpose of generating mAb, but to study the contribution of specific B cells to the overall immune response or to study the requirements for Ag-mediated activation of B cells. Ag-specific selection, however, posed a difficult challenge, as it is estimated that only one in every 10^6 B cells expresses an Ig receptor for any given antigenic epitope (Myers et al, 1986). Nevertheless, a number of approaches were developed for obtaining populations of B cells enriched for Ag-specific cells.

Three major, early approaches were utilized to enrich for specific B cells: (1) Ag was immobilized on solid matrices and specific cells adsorbed and eluted; (2) for hapten-binding B cells, haptenated red cells were used to rosette the specific B cells, followed by velocity and/or density sedimentation to purify the rosetted cells; (3) fluoresceinated Ag was bound to specific cells, which were then separated from irrelevant cells by fluorescence-activated cell sorting (FACS).

Since these early methods formed the basis for subsequent Ag-specific selection techniques, a brief review of these early

experiments will be presented first, followed by a discussion of the pertinent literature dealing with selection attempts to obtain mAb. It should be noted that the earlier experiments were done to isolate specific B lymphocytes and not hybridomas. Experiments related to monoclonal antibody technology will be described in section 1.2.

1.1.1 IMMUNOADSORBANT TECHNIQUES

The earliest attempt to purify Ag-binding cells was reported by Wigzell and Andersson (1968). These investigators passed primed lymph node cell suspensions over columns of Ag-coated glass beads and subsequently eluted the adsorbed cells by mechanical agitation. Indirect evidence for significant retention of Ag-specific cells was shown by a decreased immunocompetence of the non-binding cell population to the Ag used for coating the column. However, the retained cells were difficult to elute and showed only a 2.5-fold enrichment in specificity and a considerably decreased viability. There was also a large amount of non-specific adsorption of cells to the columns, resulting in significant cell losses. This technique was later extended to studies of non-immune cells (Wigzell and Mäkelä, 1970; Moroz and Kotoulas, 1973).

Following this beginning, immunoadsorbant systems based on polyacrylamide beads (Truffa-Bachi and Wofsey, 1970; Wofsey et

al, 1971) and agarose (Davie and Paul, 1971) were reported, in which the non-specific binding of cells was greatly reduced. Other solid-phase systems that were investigated included binding to plastic tubes (Choi et al, 1974), to poly-L-lysine-fixed antigen monolayers (Greeley et al, 1974), and to nylon fibers (Edelman et al, 1971; Rutishauser et al, 1973). However, in most cases, recovery of specific, immunocompetent cells from the bound populations remained a problem, allowing these systems to be most useful for obtaining populations of cells depleted of Ag-specific cells.

In 1973 Schlossman and Hudson described an enzyme-digestible immunoadsorbant based on Sephadex. In addition to minimal non-specific retention of cells, this method also allowed better recovery of bound cells, by solubilization of the matrix. These investigators passed mouse spleen cells through Sephadex anti-Fab columns and showed that the passed cells were specifically depleted of Ig-bearing B cells. In addition, 98% of the adsorbed B cells could be recovered in a functionally viable state after solubilization of the immunoadsorbant with dextranase.

A solid-phase immunoadsorbant system in which specific cells were adsorbed to hapten-derivatized gelatin tubes was described by Haas et al in 1974. This system had the advantage of low non-specific adherence and simple recovery of bound cells by melting the gel at 37°C. A 30-fold enrichment of

antigen-binding cells in the selected population was achieved. In 1975 Haas and Layton modified this approach to use hapten-derivatized gelatin-coated petri dishes. This method yielded up to 42% of specific, immunocompetent cells in the purified population. Various modifications of this approach produced increased purities of the selected populations (Nossal and Pike, 1978; Pike and Nossal, 1984).

In 1976 Scott reported a system which utilized anti-fluorescein conjugated to Sephadex. This technique takes advantage of fluorescent-antigen labelling as well as affinity chromatography. Immune spleen cells were mixed with fluoresceinated Ag and passed through the antifluorescein columns. Specific cells were then eluted with an unrelated fluorescent-labelled protein and shown to be fully immunocompetent. Approximately 1% of the starting cell population was isolated using this methodology, and about 30% of these selected cells were subsequently found to be Ag-binding cells (ABC). The biggest advantage of this system was that the same immunoadsorbant could be used for any antigenic system into which fluorescein could be introduced.

These immunoadsorbant techniques have led to studies where cell populations are "panned" on some form of solid-phase Ag, with the selected cells then used for generating mAb. These papers will be discussed in section 1.2.

1.1.2 RED BLOOD CELL ROSETTING TECHNIQUES

A number of different lymphoid populations will form rosettes with red blood cells (RBC), depending upon the type of red cell used, the immune status and species of the lymphocyte donor and the particular experimental methods used to prepare the rosettes. Under carefully defined experimental conditions, rosette-forming cells (RFC) are antigen-binding cells reacting specifically with an antigen on the red cell surface (Bankhurst and Wilson, 1971; Zaalberg, 1971). RFC can be fractionated from non-RFC by velocity and/or density sedimentation techniques, since the rosettes will have a higher sedimentation velocity and a greater density than free lymphoid cells.

The rosetting procedure was first described in papers by Brody and by Brody and Papermaster, in 1970. In an attempt to define the cell populations responsible for mouse immunocompetence to sheep erythrocytes, these investigators used rate zonal sedimentation to separate RFC from other spleen cells. They found that the rosetted population contained most of the Ab-forming cell precursors to sheep RBC. Their results were confirmed and expanded in later experiments (Osoba, 1970; Gorczynski et al, 1971; Sulitzeanu and Axelrad, 1973; Wilson, 1973; Tanenbaum and Sulitzeanu, 1975;).

Although these experiments were successful in providing much-

needed information about the cell populations involved in the immune response, the rosetting procedure itself was generally quite poor, with purities usually below 20%. In 1978 the rosetting procedure was modified by Kenny et al, to include a combination of density and velocity centrifugation. The two-step procedure produced 50-100% RFC from primed spleens and 20-40% RFC from virgin splenocytes. This approach was further improved by Snow et al (1983) using Percoll gradients to achieve 60-70% RFC from unprimed spleens.

A further modification of the rosetting procedure then led to two reports (Yefenof et al, 1985; Myers et al., 1986) where, by varying the extent of haptenation of the RBC used for rosetting, Ag-binding B cells with different affinities for Ag could be purified. The investigators showed that Ag-specific memory cells, virtually free of virgin cells, could be isolated.

As in the case of the immunoadsorbant procedures, the early rosetting techniques led to experiments in which rosetting was used as a means of isolating Ag-specific cells for hybridoma production. Both RBC rosetting and rosetting using metallic beads have been attempted, and these studies will be discussed in section 1.2.

1.1.3 FLUORESCENCE ACTIVATED CELL SORTING TECHNIQUES

Julius et al (1972) were the first to describe specific B cell enrichment using a fluorescence-activated cell sorter (FACS). They obtained viable and functional populations of specific cells, enriched up to 500-fold, from immune mouse spleen cell suspensions. Concomitantly, populations largely depleted of antigen-binding cells were obtained. Ag-binding cells were labelled directly with fluorescein-conjugated Ag and then isolated using a FACS. Separated populations typically contained greater than 65% fluorescent labelled cells.

In 1974 Julius and Herzenberg extended, and expanded on, these results in unprimed mice. Here, the separated population contained over 90% fluorescent labelled cells.

In 1975 Scott and Tyrer utilized a similar approach for their studies of tolerance induction. This was an *in vivo* experiment in which fluorescein labelled tolerogen (sheep gamma globulin) was injected into rats and fluorescent spleen cells were isolated at various time intervals thereafter, using a FACS. The authors found that antigen-binding cells comprised approximately 0.1% of the splenic population at all times examined (days 0-7), and that they were detectable as early as fifteen minutes after Ag injection.

This flow cytometry method seemed to hold the greatest promise for obtaining 100% pure populations of Ag-specific cells.

However, because of the low frequency of these cells, extremely long periods of sorting are often required, making this procedure impractical in some cases. As will be seen in the next section, the application of flow cytometry to Ag-specific selection for the purpose of mAb production has received only limited use.

1.2 PREVIOUS ATTEMPTS TO SELECT ANTIGEN-SPECIFIC CELLS FOR THE PURPOSE OF OBTAINING MONOCLONAL ANTIBODIES

A number of studies in recent years have attempted to improve the efficiency of specific hybridoma creation by selecting Ag-specific cells. Both pre- and post- fusion selection have been attempted, in both mouse and human systems. These experiments can be divided into five categories, based on the method used for Ag-specific selection: (1) panning; (2) RBC rosetting; (3) fluorescence-activated cell sorting; (4) immunomagnetism; (5) Ag-specific electrofusion. The literature pertaining to these techniques will now be reviewed.

1.2.1 PANNING TECHNIQUES

Panning refers to incubation of a mixed population of cells on some form of solid-phase Ag in order to isolate (or deplete) cells bearing surface receptors to that Ag from the

population. This method is an extension of the earlier immunoadsorbant techniques.

Panning has been used to isolate various subpopulations of cells. Fractionation of mouse T and B lymphocytes (Wysocki and Sato, 1978), enrichment of murine CD5 B cells (Metzger et al, 1992), and isolation of thymocyte precursor cells (Small et al, 1994) are but a few examples of the successful use of panning using plastic dishes coated with various Ag or Ab.

Panning for Ag-specific cells for the purpose of hybridoma creation has been done in at least three studies. In 1983 Winger et al utilized this technique to generate human anti-sperm Ab from peripheral blood mononuclear cells (PBMC). Petri dishes were coated with sea urchin sperm and T cell depleted PBMC were added. Selected cells were transformed with Epstein-Barr virus (EBV) in limiting dilution culture. 240 wells subsequently grew colonies and, of these, 36 were found to have antibodies in the supernatant which would bind to sea urchin sperm. Three were found to bind human sperm as well. No comparison with unselected cells was reported.

In 1993 two very interesting studies were reported by Steenbakkers et al in which panning was used to select Ag-specific cells. Both studies were based on a previous report by these same investigators (Steenbakkers, van Meel, Olijve, 1992), where a new method for the generation of human and murine B cell hybridomas was described. The method was based

on clonal expansion of single B cells in the presence of human T cell supernatant and irradiated murine thymoma helper cells. Subsequently, the clonally expanded B cells were immortalized by electric field mediated cell fusion. The investigators generated murine anti- human immunodeficiency virus (HIV) antibodies and human anti-rubella antibodies using this technique.

Expanding on this study, the authors then attempted Ag-specific selection prior to clonal expansion and electrofusion. In the first of two reports (Steenbakkers, van Wezenbeek, Olijve, 1993), murine HIV-specific spleen cells were isolated from immune mice by panning on HIV viral lysate-coated culture wells. The selected cells were clonally expanded in 96-well culture plates, using the method previously described. When cultures were sufficiently grown, supernatants were tested for anti-HIV activity. Approximately 24% of the culture wells were specific for the Ag, compared to less than 1% in the control populations (unseparated and unbound cell fractions). However, it was estimated that only 5% of the specific B cells were recovered by the panning procedure. Subsequently, six of the positive cultures were subjected to mini-electrofusions, and five of these yielded anti-HIV producing hybridomas.

In the second study (Steenbakkers, van Wezenbeek, van Zanten, 1993), human anti-cytomegalovirus (CMV) hybridomas were

generated from PBMC obtained from donors who had recently recovered from a CMV infection. PBMC were incubated on CMV-coated culture wells for selection of Ag-specific cells. The selected cells were clonally expanded in 96-well culture plates, again using the method previously described by these investigators. Between day 8 and day 12, supernatants were tested for anti-CMV activity. The purity of anti-CMV specific B cells varied from 6% to 38% in the selected populations, with an average of 0.5% in the controls (unselected B lymphocytes from the same donors). As many as twenty-two Ag-specific clones were obtained from as little as 1.5 ml donor blood. Ten Ag-specific clones were then submitted to separate mini-electrofusions, and six were successful in producing stable Ag-specific hybridomas. However the recovery of Ag-specific B cells was again very low; this time approximately 10% recovery was estimated. The authors suggest that high-affinity memory cells are preferentially selected, and this is supported by the fact that the great majority of positive B cell cultures analyzed were of the IgG class.

1.2.2 RED BLOOD CELL ROSETTING TECHNIQUES

As stated earlier, for hapten-binding B cells, haptenated red cells can be used to rosette specific B cells, with the rosettes subsequently purified by velocity and/or density

sedimentation techniques. Several studies have used this approach to isolate human Ag-specific cells for Ab production purposes.

In 1977 Steinitz et al generated anti-NNP (4-hydroxy-3,5-dinitrophenacetic acid) producing human lines from three donors. Lymphocytes were rosetted with autologous red cells coupled to NNP, and the rosettes were separated and infected with EBV. Permanent lines were established from all three donors. Rosetted lines contained 13% to 18% NNP rosetting cells, while control lines of unfractionated and rosette-deprived lymphocytes from the same donor showed no rosette-forming cells. One rosetted line was re-rosetted, resulting in an increased purity of 86%. The NNP-agglutination titer was undetectable in control lines, but showed an average titer of 256 in the rosetted lines and 1024 in the re-rosetted population. All rosetted lines secreted IgM Ab.

In 1979 Kozbor et al successfully established anti-TNP (trinitrophenyl) producing human lines by preselection of unimmunized lymphocytes with TNP-coupled erythrocytes and subsequent transformation with EBV. Non-selected cells from the same donor were also transformed to serve as controls. All resulting lines were tested for rosette and plaque formation with TNP-RBC, and the supernatants were titrated for anti-TNP activity. Using the technique of Osoba (1970), rosette-forming cells increased from 0.1% in the unselected population to 75%

after three cycles of rosetting. In parallel, plaque-forming cells (PFC), which were undetectable in the primary population, accounted for 30% of the rosetted cells, and the TNP Ab titer in the supernatants increased from 16 to 512. The TNP Ab-producing lines established from the rosetted populations produced specific, polyclonal IgM Ab.

In 1983 Winger et al used rosetting to isolate Ag-specific cells from unimmunized human donors. Chicken RBC, neuraminidase-treated human RBC, and calf thymus DNA-coupled sheep RBC were used for rosetting. The selected populations were transformed with EBV and cultured by limiting dilution. Antibodies to chicken erythrocytes, to neuraminidase-treated (but not untreated) human RBC, and to DNA were found. No comparisons with unselected populations were reported.

Koskimies (1980) used this rosetting approach to establish a human line producing anti-Rhesus(D) Ab. Ag-specific lymphocytes from an immunized donor with a high titer of anti-D were preselected by rosetting with Rh-positive erythrocytes prior to EBV infection. Continuously growing lines from both the selected and deprived fractions were obtained. The selected line produced anti-D Ab while the deprived line did not. However, the selected line also produced nonspecific Ab. By re-rosetting, the specific anti-D Ab was enriched, with the Ab titer increasing from 8 to 128.

1.2.3 FLUORESCENCE ACTIVATED CELL SORTING TECHNIQUES

Isolation of Ag-specific cells using fluorescence-activated cell sorting, for the purpose of mAb production, has received very limited use. This is somewhat surprising given its widespread use in other areas as a means of selecting rare cells. However at least two studies have been reported, and they both show favorable results.

Parks et al (1979) performed practical demonstrations utilizing flow cytometry, to show that Ag-specific hybridomas could be isolated from a mixture of hybridomas. In several experiments they mixed cells producing mAb to mouse Ig allotypes, at a frequency of 1 in 500, with hybridomas producing some other Ab. The rare cells were recovered by labelling with fluorescent microspheres coupled to the appropriate myeloma protein Ag, followed by sorting of the brightly labelled cells using the flow cytometer. Selected cells were sorted individually into microculture wells. In each case, the number of clones secreting specific Ab in the sorted population showed an enrichment factor of about 250 over the original 1 in 500 mixture.

In 1986 Casali et al used a modification of this approach to isolate Ag-specific B cells from human subjects prior to EBV transformation. In these experiments, purified Ag (either thyroglobulin or tetanus toxoid) was biotinylated and

incubated with purified B lymphocytes from healthy donors. The cells were then reacted with fluorescein isothiocyanate (FITC)-avidin and analyzed by flow cytometry. It was found, in repeated experiments, that 5%-10% of cells showed a high degree of fluorescence. These cells were sorted and the positive and negative fractions collected and transformed. The cells were then plated in 96-well culture plates at approximately 4000 cells per well. All wells from the positive fraction were subsequently found to be producing high levels of Ab to the Ag, while fewer than 6% of wells from the negative fraction produced any detectable Ab. The investigators cloned some of the positive wells to yield cell lines producing mAb.

1.2.4 IMMUNOMAGNETISM

During the last decade, immunology has seen the introduction of a technology in which magnetic fields play an integral role. Metallic beads coated with antibodies or other proteins can be used to isolate specific cells from mixed populations. Cells bearing receptors to the protein will form rosettes with the metallic beads, and isolation of these specific cells is achieved easily and rapidly by placing the cell suspension against a magnet and pouring off the unbound cells.

This technology was made possible by the work of Ugelstad et

al (1983), who developed a method for preparing metallic beads, comprised of an iron core and a polymer shell, of uniform size, density and iron content. Functional groups could be incorporated onto the surface of the beads, which could then be modified for physical adsorption or covalent coupling of proteins.

Metallic beads coated with a variety of antibodies and other proteins, as well as beads to which a desired protein can be readily attached, are now commercially available, and this has allowed widespread use of this technology. In addition to Ag-specific selection, some of the many ways these beads have been used include the removal of tumor cells from bone marrow (Treleaven et al, 1984; Vredenburgh et al, 1991), the depletion of T cells for bone marrow transplantation (Gee, 1989), isolation of specific cell populations for functional assays (Hovdenes et al, 1989; Tjernlund et al, 1988), and as a tool for cell purification for serologic tissue typing (Vartdal et al, 1986; Hansen et al, 1987).

Metallic beads have been used in several studies to isolate Ag-specific cells for the purpose of both human and murine hybridoma creation. In 1988 Egeland et al coated metallic beads with rabbit IgG in order to rosette rheumatoid factor B lymphocytes from peripheral blood of patients with rheumatoid arthritis. The principle here is that rheumatoid factor specifically recognizes the Fc fragment of rabbit IgG. Thus

beads coated with IgG will rosette B cells expressing rheumatoid factor. The rosetted cells were then transformed with EBV. Unfractionated B cells from rheumatoid arthritis patients were used as controls. In several experiments, more than 90% of culture wells from the rosetted population produced rheumatoid factor, compared to less than 6% in the controls.

A report by Glaser et al in 1990 describes a combination of magnetic separation followed by electrofusion of human peripheral blood cells. In this experiment peripheral blood mononuclear cells were reacted with beads coated with anti-CD19 Ab. Selected and unselected cells were then electrofused with the heteromyeloma line CB-Fu2, to form hybrids. In unseparated PBMC, 7% CD19+ and 68% CD3+ cells were detected using flow cytometry. In the population selected using anti-CD19 coated beads, the contamination with CD3+ cells was below 2%; however, only about 2% of the starting population of PBMC could be recovered from the anti-CD19 coated beads. The electrofusion procedure was successful in producing hybridomas, even when the number of cells placed in the fusion chamber was very low. A fusion frequency of 1-15 x 10^{-5} was observed for unseparated PBMC, while the separated fraction showed a fusion frequency of 5-50 x 10^{-5}. The investigators then determined the percentage of cytoplasmic Ig-positive cells in both populations. In each experiment, significantly

more hybridomas were Ig-positive in fusions of CD19+ cells compared with unseparated cells ($P<0.005$).

In 1993 Lundkvist et al utilized this technique while attempting to make human mAb against Puumala·virus. Human spleen cells were obtained from a splenectomized patient, and virus-coated beads were used for selection. Virus coating was accomplished using virus-specific murine mAb for indirect binding of viral glycoproteins to the magnetic beads. Three strategies were employed: (1) unselected spleen B cells were EBV transformed (2) unselected cells were transformed, grown in batch culture for 21 days and then selected with beads (3) spleen cells were selected with beads prior to transformation. In all cases, transformed cells were cloned by limiting dilution, and growing cultures were screened for specific Ab production. No positives were found in the control group (unselected spleen cells). Several Ag-specific hybridomas were found in the population selected with beads 21 days post-transformation, but these were lost following cloning. Positive results were obtained when spleen cells were preselected by viral glycoprotein-coated metallic beads. Twenty out of 21 growing culture wells in this population were positive against the virus. Four of these wells were successfully cloned to ensure monoclonality and produced specific IgG Ab. Further testing strongly indicated that the four mAb were all derived from the same original clone. Three

of these monoclonal lymphoblastoid cell lines were fused with a human/mouse heterohybridoma to generate stable, Ag-specific hybridomas.

In 1994 Oshiba et al utilized Ag-coated immunomagnetic beads to isolate Ag-specific B cells from the peripheral blood of immunized donors. B cells separated with tetanus toxin- or keyhole limpet hemocyanin-coated beads were transformed with EBV. The amount of specific antibody produced by this rosetted population was compared to that of unfractionated and nonrosetting transformed populations. The rosetted populations were found to contain significantly more specific antibody than either of the other two populations; 700-1500 units per ml of specific Ab was reported for the rosetted populations compared to 0-70 units for the control populations.

In addition to these human studies, immunomagnetism has also been utilized in the generation of murine mAb. Three such studies will be described here; two of these used Ag-coated beads to select specific hybridomas from bulk cultures derived from PEG-mediated fusions (and are therefore similar to the experiments done in this study), while the third method used bead selection to isolate specific B splenocytes prior to fusion.

In the first of these reports, Ossendorp et al (1989) immunized mice with thyroglobulin, fused the spleen cells with SP2/0 cells using PEG, and grew the resulting population as a

bulk culture in selective HAT medium. One week after fusion, viable hybridoma cells were counted and divided into two groups. One was incubated with thyroglobulin-coated metallic beads and the other with uncoated beads. Immunomagnetism allowed separation of magnet-bound and non-bound fractions for each group. These four populations were then cultured in 96-well plates, and supernatants from growing hybrids were subsequently tested for anti-thyroglobulin activity. The magnet-bound population from the uncoated beads produced only six cultures, none of which showed any specific Ab activity. 87 growing wells were found in the magnet-bound population from thyroglobulin-coated beads, and 95% of these produced anti-thyroglobulin Ab, all of the IgG isotype. The percentage of thyroglobulin positive wells in the non-bound population using Ag-coated beads was approximately 15%, and this was slightly lower than that in the non-bound population using uncoated beads.

These investigators also determined the frequency of Ag specific hybridomas in the three cell populations resulting from selection using thyroglobulin-coated beads; namely, the original population, the magnet-bound population and the non-bound population. The frequency of anti-thyroglobulin hybrid cells in the original bulk population was found to be 1 in 500; in the non-bound population, 1 in 1030; and in the magnet-bound cells, 1 in 1.7. This was an enrichment of 300-

fold. Recovery of Ag-specific cells was estimated at 50%. Additional experiments in this study showed that stable rosette formation was correlated with the affinity of the Ab produced by the hybridoma, indicating that this method results in selection of high affinity hybridomas. The authors suggest that this method may be particularly useful for Ag of low immunogenicity. They also point out that a substantial amount of pure Ag is required to coat the beads, making it unsuitable for many systems.

A similar experiment was performed by Bennick et al in 1993. In this case fragment D dimer (DD) from plasminolysed fibrin was used as Ag. Immune murine spleen cells were subjected to PEG-induced fusion, and the resulting cells were either cloned directly in 96-well plates in the conventional way, or grown as a bulk culture for ten days and then selected using Ag-coated metallic beads. Beads were coated by first incubating with a mAb to DD and then with DD. The magnet-bound population was then plated in 96-well plates. Supernatants from growing wells from both the conventional fusion and the selected fusion were then tested for specific Ab production. Six experiments were done; three using conventional methodology and three using bead selection. The percentage of viable cultures in each group was similar. However, specific clones were obtained in 10%, 19%, and 30% of wells using the conventional fusion procedure and in 40%, 52%, and 79% of the

wells resulting from bead selection. Thus the bead selection procedure resulted in a percentage of viable clones that compared well with a successful, conventional fusion, but with a percentage of specific clones 3-4 times higher than that obtained using the conventional approach. The investigators note that, in their efforts to obtain hybridoma clones of interest, implementation of the bead selection method significantly reduced costs and time.

A final study is contained in the report of Steenbakkers et al (1993), which was partly described earlier. In addition to panning for HIV-specific B cells (discussed in section 1.2.1), these investigators also used HIV-coated metallic beads and immunomagnetism to select Ag-specific cells from the same mice prior to clonal expansion and electrofusion. Approximately 7% of culture wells from the magnet-bound population were subsequently found to be secreting Ag-specific Ab, compared to less than 1% in the controls (unselected cells). This enrichment was less than that obtained by panning (24%). However, the recovery of Ag-specific cells, which was estimated at 5% for panning, was improved using this bead selection method (estimated at 17%). Eight of the positive colonies were then electrofused, and all eight fusions produced stable, Ag-specific hybridomas.

1.2.5 AG-SPECIFIC ELECTROFUSION

Electrofusion offers an alternative to PEG as a means of obtaining somatic cell hybridization and thus immortalization. Vienken and co-workers (1982), reported that a variety of cell types can be fused efficiently by this method. Advantages offered by pulsed electric field (PEF) methods over PEG induced fusions include a higher fusion efficiency, a decreased number of cells required for efficient hybridoma formation, and a wider application (in that human cells can also be fused with high efficiency (Meng et al, 1988)). Studies comparing PEG fusion with electrofusion show fusion frequencies up to 80-fold higher using the latter technique (van Duijn et al, 1989). However, in early electrofusion attempts to obtain mAb, the frequency of Ag positive wells was quite low, again indicating a need for Ag-specific selection. Several studies have already been discussed in which electrofusion was used on Ag-specific cells preselected by panning (Steenbakkers et al, 1993, 1993a) and by immunomagnetism (Steenbakkers et al, 1993; Glaser et al, 1990). In addition, a method has been developed whereby an avidin-biotin bridge links Ag-specific cells to myeloma cells for the electrofusion procedure. These selective electrofusions offer significantly increased fusion efficiencies as well as high selectivity. This avidin-biotin

selection technique, described briefly below, is explained fully in a review article by Tsong (1993).

This method was first described by Lo et al in 1984. In these experiments, Ag was covalently conjugated to avidin and mixed with immune murine spleen cells. This mixture was then incubated with biotinylated myeloma cells. An intense electric field was applied to this bulk cell suspension, producing selective fusion of cells in contact; ie, of myeloma cells bound to Ag-specific B cells by the avidin-biotin bridge. Using this procedure these investigators produced mAb to rat lung angiotensin-converting-enzyme, enkephalin convertase and bradykinin. Although only a very small number of hybrids resulted from each fusion (<10 in most cases), 100% were found to be Ag-specific. Monoclonal antibodies were shown to be of high affinity, and the great majority were of the IgG class, even when only a primary immunization had been given.

In 1986 Wojchowski et al modified this technique such that biotin was attached to both the Ag and the myeloma cells, with streptavidin bridging biotinylated myeloma cells to those B cells bearing biotinylated Ag. The advantage offered by this modification was that it obviated the need to covalently modify avidin, making the procedure much easier to perform. These investigators produced mAb to KLH from immunized mouse splenocytes. Thirteen hybridomas resulted from the electrofusion, and all were found to be secreting KLH-specific

Ab of high affinity.

This procedure has since been used in several studies for the production of mouse mAb (Hewish and Werkmeister, 1989; Tomita and Tsong, 1990; Werkmeister et al, 1991). Each time, the yield of Ag-specific hybridomas was considerably increased compared to conventional fusion methods. The experiments of Hewish et al in 1989 were especially interesting in that Ag of both high and low immunogenicity were utilized and, in both cases, 100% of hybrids resulting from selective electrofusions were found to be Ag-specific.

The study of Werkmeister et al in 1991 was also very interesting. In these experiments mice were immunized with highly immunogenic potato virus coat protein. Two groups of mice were used; group 1 was immunized with coat protein from potato virus Y, while group 2 was immunized with a mixture of coat proteins from five distinct potato viruses. Sera from both groups reacted with each of the five viruses, indicating responses to both specific and common epitopes of the viral coat protein. Two well-defined peptides were then used for selective electrofusions, in order to preferentially select B cells making Ab against distinct epitopes. Peptide-1 was selected from a highly specific region of potato virus Y and was used for the group 1 mice. Peptide-2, from a highly conserved region of one of the other potato viruses, was used for the group 2 mice. Both peptides were biotinylated, as were

the myeloma cells. Streptavidin was again used as the bridging agent. Electrofusions with peptide-1 produced eleven hybridomas, all of which secreted mAb totally specific for potato virus Y. Ninety hybridomas were generated using peptide-2 and, again, 100% were found to be secreting Ag-specific Ab. Fourteen of these ninety hybrids were further studied, and mAb from twelve showed reactivity against all five potato viruses. The other two reacted with coat protein from three and four of the five viruses.

From the studies presented in this review, it is clear that Ag-specific selection techniques offer an attractive alternative to conventional fusion methodology. Such techniques can, in principle, drastically reduce the amount of time and labor that is consumed in mAb technology. However, it must be remembered that considerable time, effort and cost may be expended in implementing these selection methods. Therefore, they may not be suitable in all cases, particularly when the Ag of interest is highly immunogenic. This technology may prove to be especially useful for human mAb production, where conventional fusion technology has been highly unsatisfactory.

Several different selection techniques have been described, each having advantages and disadvantages. The method of choice will depend, to some extent, on the precise experimental

conditions being used. For the experiments in this thesis, panning and immunomagnetic selection were the methods chosen.

1.3 AIMS AND OBJECTIVES

1.3.1 OVERALL OBJECTIVES

The experiments described in this thesis stem from a larger project that was undertaken in our research laboratory; namely the creation of hybridomas secreting mAb to human leukocyte antigen (HLA) class II DP molecules. The main reason for this endeavor was to make serological HLA-DP matching of donor-recipient transplantation pairs possible.

With the discovery of the DP system (Shaw et al, 1980), it was theorized that extended matching to include DP Ag would improve the success rate of organ transplantation, particularly bone marrow transplantation (BMT). However, typing for DP was performed using the primed lymphocyte typing (PLT) test, which is cumbersome, time-consuming, and not easily adapted for use in routine tissue typing laboratories. For this reason, donor-recipient matching for HLA-DP was not included in the selection process for BMT. However, serological typing reagents were highly desirable.

In 1989 our research laboratory, headed by Dr. William Marshall, began a project with the goal of creating mouse mAb

to polymorphic determinants on DP molecules, for tissue typing use. The fact that HLA-DP could be recognized serologically had already been established by Heyes et al (1986), who made a mouse mAb to DP, and by Johnson (1986), who raised an alloantiserum in a human volunteer by deliberate immunization. Following these reports, however, very little progress was made in this area, despite the efforts of many laboratories. A possible explanation for this lack of success was offered by studies which showed that: (a) DP accounts for a low proportion of the class II molecules expressed on the cell surface, and (b) surface DP molecules are relatively unstable (Eckels et al, 1986; Johnson et al, 1986).

At the time this project was begun, nineteen DP specificities had been defined (Begovitch et al, 1989). These 19 alleles were differentiated by polymorphisms in six hypervariable regions on the beta chain. Very few allele-specific sequences were identified; rather, the DPβ alleles appeared to result almost exclusively from the "shuffling" of a limited number of sequence motifs in the six regions of variability. Therefore, it was realized that serological typing for DP would be more complex than typing for other HLA regions, requiring antibodies specific for epitopes rather than allele-specific antibodies. The possibility of additional alleles, not yet discovered, was also realized, since the number of beta alleles possible using this "epitope shuffling" process

is very large. (Indeed, at the present time, at least 55 DP alleles have been defined (Moonsamy et al, 1994).)

For these reasons we decided that, in addition to conventional fusions, several other approaches would be attempted, in an effort to boost the chances of success. At the time, conventional fusions to produce anti-DP mAb in our laboratory were showing limited success, and we hoped that the experiments described in this thesis would improve the efficiency of DP-specific mAb production.

1.3.2 SPECIFIC OBJECTIVES

The aim of the work done in this thesis was to devise and test methods for increasing the efficiency of Ag-specific hybridoma creation, by post-fusion selection of specific hybrid cells. The specific objectives were as follows:

(i) to study the kinetics of post-fusion bulk cultures, including their treatment with HAT selective medium, in order to determine the optimal time to attempt selection.

(ii) to experiment with various selection procedures to determine if any could produce significant enrichment of Ag-specific hybridomas.

(iii) to develop secure methods for culturing, cloning, and studying the selected cell populations.

(iv) to evaluate the potential of post-fusion selection

techniques, by comparison with standard random fusion procedures.

2.0 MATERIALS AND METHODS

2.1 FUSIONS

All fusions performed in this study (15 in total) were done using immune mouse splenocytes and SP2/0 myeloma cells, with polyethylene glycol (PEG) as the fusogenic agent. Mice were immunized with mouse fibroblast cells transfected with human DP genes.

2.1.1 MICE

All mice used in these experiments were either C3H or F1 (Balb/c x C3H) mice.

C3H and Balb/c mice were obtained from Charles River Canada Inc., St. Constant, Quebec.

F1 mice were bred in local animal facilities, by crossing Balb/c males with C3H females.

Table 1 lists the strain, sex, and approximate ages of the mice used in each of the 15 fusions reported in this thesis.

2.1.2 IMMUNOGENS

Five transfectant cells were used as immunogens in these experiments, each expressing a different DP type. Table 2

TABLE 1: SUMMARY OF MOUSE STRAIN, SEX, AND AGE (AT TIME OF FUSION) FOR EACH FUSION DESCRIBED IN THIS THESIS

FUSION NUMBER	STRAIN	SEX	AGE (MONTHS)
1	F1	M	2.5
2	F1	F	4.0
3	C3H	F	4.0
4	F1	F	3.5
5	C3H	F	5.5
6	F1	M	4.5
7	C3H	F	6.0
8	F1	F	5.0
9	F1	F	3.5
10	F1	F	5.0
11	F1	F	6.0
12	F1	F	6.0
13	C3H	F	5.0
14	C3H	F	12.0
15	F1	M	12.0

TABLE 2: SUMMARY OF TRANSFECTANT CELLS USED FOR IMMUNIZATIONS

TRANSFECTANT NAME	DP SPECIFICITY	DP ALLELES EXPRESSED	SOURCE
DP8304	DP 4.1	DPα1:0101 DPβ1:0401	11th IHW
DP8305	DP 4.2	DPα1:0101 DPβ1:0402	11th IHW
DP8306	DP 9	DPα1:0201 DPβ1:0901	11th IHW
DP1601	DP 16	DPα1:0101 DPβ1:1601	MUN*
DP0202	DP 2.2	DPα1:0101 DPβ1:0202	MUN*

IHW = International Histocompatibility Workshop

* Memorial University of Newfoundland; Lui and Younghusband, unpublished

lists these cells, along with their DP specificity, the DP alleles expressed, and the source of each.

2.1.2A GROWTH MEDIUM

All transfectants were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Canadian Life Technologies, Burlington, Ontario), supplemented with:

-10% heat inactivated fetal bovine serum (FBS) (Gibco BRL);

-2mM L-glutamine (Gibco BRL);

-50 units/ml penicillin, 50 μg/ml streptomycin (Gibco BRL);

-$5x10^{-5}$M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO).

(This medium will be called 10% DMEM for the remainder of this thesis.)

In addition to the above reagents, the appropriate selective agent was added to the 10% DMEM, based on the marker gene that had been co-transfected with the DP genes.

Three of the immunizing transfectant cells were obtained from the 11th International Histocompatibility Workshop (Inoko et al, 1992) (see Table 2). These cells had been made by co-transfecting the pNeo gene with the DP α and β genes. The selective agent geneticin (G418) (Gibco BRL), at a concentration of 0.25 mg/ml, was thus incorporated into the 10% DMEM for culture of these cells.

The other two transfectants were made locally, by exchanging

the second exon of a DPβ1:0201 gene (coding for amino acids 1-90) with portions of other DPβ chain genes, derived by amplification of the DNA using the polymerase chain reaction (PCR). For these two transfectants, the thymidine kinase (tk) gene had been co-transfected with the DP α and β genes. Thus selection and maintenance required HAT selective medium. These selective agents were incorporated into the 10% DMEM, for culture of these two cells, as follows:

-hypoxanthine (Sigma) at 1.50 mg/ml;

-aminopterin (Sigma) at 0.02 mg/ml;

-thymidine (Sigma) at 0.50 mg/ml.

2.1.2B TRANSFECTANT GROWTH

The cells were grown at 37°C, in 5% CO_2, in 10 cm circular tissue culture dishes (Becton Dickinson, Lincoln Park, New Jersey). When confluent growth was reached (determined microscopically), the cells were harvested by incubating at 37°C with 0.25% trypsin (Gibco BRL) in phosphate buffered saline (PBS) (3ml/plate) for 10-15 minutes. This causes the cells to become non-adherent. Trypsin was then removed by washing the cells twice in 10% DMEM (all centrifugations were done at 500 x *g* for 10 minutes unless otherwise stated). Following harvesting, cells were left in ordinary bacteriological Petri dishes (to which they do not stick) for

one to four days, at a density not exceeding $2x10^6$/ml.

2.1.2C DETERMINATION OF DP EXPRESSION ON TRANSFECTANT CELLS

Before using these cells for immunizations, it was necessary to ensure adequate DP expression. This was done by flow cytometry, using the mAb NFLD.M67, which was made in this laboratory and has been determined to react with a monomorphic epitope on DP molecules. Supernatant fluid, obtained by overgrowing the NFLD.M67 hybridoma cells in a 75 cm^2 tissue culture flask (Becton Dickinson), in 10% DMEM, for 3-4 weeks, was the source of this Ab.

2.1.2C.a REAGENTS FOR FLOW CYTOMETRY

i. AZIDE BUFFER

To make one liter of azide buffer, 1 ml FBS and 0.1 gm sodium azide (BDH Chemicals, Toronto, Ontario) were dissolved and then made up to 1 liter in PBS.

ii. CONJUGATE (SECOND ANTIBODY)

R-phycoerythrin-conjugated goat anti-mouse (GAM) IgG (Jackson ImmunoResearch, West Grove, PA) was diluted in azide buffer, using a previously determined optimum dilution.

iii. PARAFORMALDEHYDE

5% paraformaldehyde (BDH Chemicals) in PBS.

2.1.2C.b PROCEDURE FOR FLOW CYTOMETRY

-for each transfectant being tested, 250,000 cells were added to each of two 12 x 75 mm polystyrene tubes (Fisher Scientific, Montreal, Quebec);
-cells were washed x1 with 3 ml azide buffer;
-25 μl of 10% DMEM was added to one tube (negative sample) and 25 μl NFLD.M67 (positive sample) to the other tube;
-tubes were incubated at 4°C for 30 minutes;
-cells were washed x2 with azide buffer;
-25 μl conjugate was added to both tubes;
-tubes were incubated at 4°C for 30 minutes;
-cells were washd x2 with azide buffer;
-cells were resuspended in 200 μl paraformaldehyde and analyzed in a FACStar Plus flow cytometer.
Histograms of cell number vs fluorescence were obtained, and a mean channel shift of >100 was required for cells to be used for immunizations.

2.1.3 IMMUNIZATIONS

Transfectant cells to be used for immunization were:

-collected from their Petri dishes into 50 ml centrifuge tubes;

-counted using a haemocytometer;

-washed x3 in PBS;

-resuspended in PBS for immunization.

Table 3 lists the immunogen and the immunization schedule for each of the 15 fusions described in this thesis. Many of these fusions were experimental procedures aimed at optimizing the conditions to achieve the highest possible yield of specific Ab. For this reason, the immunization schedule varied between fusions, with regard to the number of cells injected, the number of immunizations given, and the length of time between immunizations.

2.1.3A PRIMARY IMMUNIZATIONS

Primary immunizations were all done subcutaneously (SC), using complete Freunds adjuvant (CFA) (Sigma). The required number of cells was resuspended in 0.3 ml of PBS and emulsified with 0.3 ml CFA, using two 3 ml syringes connected via a double-ended fitting. The emulsion was then injected subcutaneously, using a 23-gauge needle, in two or three sites on the mouse's back.

TABLE 3: IMMUNIZATION SCHEDULES FOR THE 15 FUSIONS

FUSION	IMMUNOGEN	# OF CELLS ($X 10^6$)	MODE OF INJECTION	TIME (WKS) BETWEEN
1	DP8304	1°: 20 BOOST: 10	SC IV	. 3
2	DP8305	1°: 25 BOOST: 25	SC IV	4
3	DP8305	1°: 25 BOOST: 25	SC IV	4
4	DP8306	1°: 25 BOOST: 30	SC IV	7
5	DP8306	1°: 25 BOOST: 35	SC IV	12
6	DP8306	1°: 20 BOOST: 100	SC IV	10
7	DP8305	1°: 25 2°: 20 BOOST: 30	SC IP IV	4 5
8	DP8306	1°: 25 BOOST: 35	SC IV	4
9	DP8306	1°: 22 BOOST: 35	SC IV	6
10	DP8304	1°: 20 BOOST: 30	SC IV	5
11	DP0202	1°: 25 2°: 23 BOOST: 30	SC IP IV	10 4
12	DP0202	1°: 25 2°: 23 BOOST: 30	SC IP IV	10 4
13	DP1601	1°: 20 BOOST: 45	SC IV	5
14	DP8305	1°: 40 BOOST: 30	SC IV	36
15	DP8304	1°: 25 2°: 32 BOOST: 22	SC IP IV	8 16

1° = Primary immunization 2° = Secondary immunization

2.1.3B BOOST IMMUNIZATIONS

Boost immunizations were all done intravenously (IV), three days prior to fusion. The required number of cells was resuspended in 0.5 ml PBS, and injected into a tail vein using a 29-gauge needle.

2.1.3C SECONDARY IMMUNIZATIONS

Secondary immunizations were done for four of the fifteen fusions (see Table 3). The required number of cells was resuspended in 0.5 ml PBS and injected intraperitoneally (IP) using a 26-gauge needle. The IV boost was thus a tertiary immunization for these four fusions.

2.1.4 CELL FUSION PROCEDURE

2.1.4A REAGENTS FOR CELL FUSION

i. SERUM-FREE (SF) DMEM

DMEM supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin.

ii. ALKALINE DMEM

Serum-free DMEM adjusted to pH 8.0 using 5N NaOH.

iii. POLYETHYLENE GLYCOL (PEG)

A 50% PEG (mw 3350) (J.T. Baker Co., Phillipsburg, New Jersey) solution was used for all fusions. It was prepared as follows:

-one gm of PEG was melted by autoclaving, and cooled to approximately 50°C;

-one ml of alkaline DMEM (prewarmed to 37°C) was added;

-the solution was mixed well and left at 37°C until ready for use.

iv. T-CELL AB-COATED BEADS

Reagents:

-metallic beads coated with sheep anti-rat IgG (Dynal Inc., Great Neck, New York);

-Rat anti-mouse T cell Ab: this Ab was obtained by overgrowing the TIB 104 cell line (American Type Culture Collection, Rockville, Maryland) in a 75 cm^2 tissue culture flask (Becton Dickinson), in 10% DMEM, for 3-4 weeks, and harvesting the supernatant fluid. TIB 104 is a hybridoma which secretes a rat IgG2a anti-mouse Ab, reactive with the lymphocyte differentiation Ag Lyt 1 of mice (Ledbetter et al, 1979);

-1% FBS/PBS: 1 ml FBS added to 99 ml PBS.

Method:

-4 x 10^8 beads were removed and added to a 15 ml conical centrifuge tube;

-beads were washed x2 by resuspension in 7 ml 1% FBS/PBS. The beads were collected by placing the tube in a magnetic particle concentrator (Dynal Inc.) for 1-2 minutes, followed by pouring off the fluid while the tube was still retained in the magnet; this will be referred to as the magnet/washing procedure for the remainder of this thesis;
-after the second wash, 7 ml of overgrown TIB 104 supernatant was added to the beads;
-the tube was rotated overnight at 4°C;
-beads were collected using the magnet, and the supernatant was discarded;
-beads were washed x4 by resuspension in 7 ml 1% FBS/PBS, rotating for 30 minutes at 4°C, and collecting the beads using the magnet;
-beads were stored in 8 ml 1% FBS/PBS at 4°C until ready for use.

v. MOUSE FEEDER CELLS

For these fusions, mouse RBC and mouse spleen cells were incorporated into the hybridoma medium as feeder cells to facilitate growth of the hybridomas. They were obtained from an unimmunized mouse of the same strain as the one being used for fusion, as follows:
-the mouse was anesthetized with ether and killed by cervical dislocation;

-the mouse was immersed in 70% ethanol for a few seconds, then pinned to a styrofoam board, and cut open using aseptic technique;

-the RBC were obtained by cardiac puncture, using a 1 ml tuberculin syringe, and immediately resuspended in 10 ml serum free DMEM in a heparinized vacutainer tube (Becton Dickinson) containing 143 USP units of sodium heparin;

-the spleen was removed and the spleen cells collected in the same way as the fusion spleen cells (see section 2.1.4B.a). The feeder spleens were then resuspended in 6 ml of 10% DMEM in a 15 ml centrifuge tube and irradiated, using 800 rads (Newfoundland Cancer Clinic, Health Science Center, St. John's), to prevent subsequent growth;

-feeder RBC and feeder spleens were then washed x2 in 10% DMEM, resuspended in 10 ml 10% DMEM, and counted.

vi. HYBRIDOMA MEDIUM

DMEM supplemented with:

-20% FBS (Gibco BRL);

-2mM L-glutamine (Gibco BRL);

-50 units/ml penicillin, 50 μg/ml streptomycin (Gibco BRL);

-$5x10^{-5}$M 2-mercaptoethanol (Sigma);

-irradiated mouse feeder spleen cells at approximately $1x10^{6}$/ml;

-mouse feeder RBC at approximately $2x10^{7}$/ml.

vii. FUSION HAT MEDIUM

A stock solution 100x concentrated was made as follows:

-136 mg hypoxanthine (Sigma);

-1.76 mg aminopterin (Sigma);

-38.8 mg thymidine (Sigma).

The above reagents were dissolved in 80 ml distilled water (dH_2O). A few drops of 5N NaOH were added to facilitate dissolving, and the solution was made up to 100 ml. Sterilization was achieved by forcing the solution through a syringe mounted with a 0.22μm Millipore filter (Millipore Corporation, Bedford, MA).

5 ml aliquots were stored at -20°C.

Aliquots were thawed as required and added to 20% DMEM. To make 1x HAT medium, 5 ml stock was added to 495 ml 20% DMEM. To make 2x HAT medium, 5 ml was added to 245 ml 20% DMEM.

viii. FUSION PARTNER CELLS

The mouse myeloma line SP2/O-Ag14 (Shulman et al, 1978) was a gift from Dr. Robert Weaver, Vancouver, and was the fusion partner for all fusions performed in this study. These cells do not secrete immunoglobulin chains and they are deficient in the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRTase). Therefore, they are sensitive to HAT medium.

The cells were grown in 75 cm^2 tissue culture flasks, in 10% DMEM, at a density not exceeding $2x10^5$/ml, with a viability

greater than 90%.

2.1.4B FUSION PROCEDURE

2.1.4B.a IMMUNE SPLEEN CELLS

Immune spleen cells for fusion were obtained as follows:
-the immunized mouse was anesthetized with ether and killed by cervical dislocation;
-the mouse was immersed in 70% ethanol, pinned to a styrofoam board, and cut open using aseptic technique;
-the spleen was removed and placed in a Petri dish containing 10 ml of serum-free DMEM;
-the cells were teased from the organ using a pair of sterile 21 gauge hypodermic needles mounted on syringe barrels and bent to 90°;
-the "empty" spleen capsule was discarded, and the resuspended cells were pipetted into a 50 ml conical centrifuge tube;
-the Petri dish was rinsed with 10 ml of serum-free DMEM, which was added to the 50 ml tube;
-clumps were allowed to settle for approximately five minutes, and the cell-rich supernatant was pipetted into another 50 ml tube;
-the cells were centrifuged, washed once in serum-free DMEM, resuspended in 7 ml 10% DMEM, and counted.

2.1.4B.b T-CELL DEPLETION OF IMMUNE SPLEEN CELLS

Immune spleen cell suspensions were subjected to T-cell depletion prior to fusion, as follows:

-the appropriate number of T-cell Ab-coated beads (ratio of 3 beads per spleen cell) were transferred to a 15 ml centrifuge tube;

-the tube was placed in a magnetic holder for 1-2 minutes and the fluid was poured off;

-the spleen cells, resuspended in seven ml of 10% DMEM, were added to the beads;

-the tube was rotated for 15 minutes at room temperature;

-the tube was placed in the magnetic holder for 1-2 minutes, and the T-cell depleted cell suspension was poured off into a 50 ml tube;

-the metallic beads were washed once by resuspension in 7 ml 10% DMEM followed by another application of the magnet. The supernatant was poured off into the same 50 ml tube as above;

-the beads were then discarded and the T-cell depleted cell suspension was counted.

2.1.4B.c PRE-FUSION PREPARATION OF CELLS

-SP2/O cells were counted and the appropriate number (ratio of one SP2/O for every 10 spleen cells) transferred to a 50 ml

centrifuge tube;

-SP2/O cells and immune T-cell depleted spleen cells were centrifuged and washed x2 with serum-free DMEM;

-SP2/O cells and immune spleen cells were combined in one 50 ml centrifuge tube and washed x2 with serum-free DMEM;

-after the last wash, the tube was inverted on sterile gauze and allowed to drain, to ensure complete removal of medium.

2.1.4B.d POLYETHYLENE GLYCOL INDUCED CELL FUSION

-1 ml 50% PEG was slowly added to the cell button, over a timed 2 minute period, while maintaining constant rotation (by hand) of the tube. A 2 ml pipette was used;

-2 ml alkaline DMEM was added in the same manner, again over a 2 minute period;

-8 ml alkaline DMEM was then added, using a 10 ml pipette, over a 3 minute period;

-the suspension was centrifuged at 300 x *g* for 8 minutes;

-the supernatant was decanted without disturbing the cell button;

-10 ml serum-free DMEM was vigorously added and the cells were centrifuged again;

-the supernatant was decanted, and the fused cells were gently resuspended in hybridoma medium, at a concentration of approximately 1.3×10^6 immune spleen cells per ml.

2.1.4B.e CELL CULTURING OF FUSED POPULATION

For the fusions described in this thesis, the majority of the post-fusion cell population was plated immediately, following conventional fusion methodology, into 96-well, flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark); 100 μl of the post-fusion suspension (containing 1.3 x 10^6 immune spleen cells/ml) was added to each well. The fusion procedure is well established in this laboratory, and these conditions had already been optimized to allow oligoclonal hybrid growth.

For the purpose of this study, a portion of the fused cell suspension was grown as a bulk culture, for subsequent Ag-specific selection. 25 ml aliquots of the fused suspension were added to 75 cm^2 tissue culture flasks (Becton Dickinson), for bulk hybrid growth.

Table 4 lists the total number of immune spleen cells used in each fusion, the percentage of each fusion that was plated in the conventional manner, and the selection method used (immunomagnetism or panning) on the remaining bulk culture, for each fusion experiment in this study.

TABLE 4: TOTAL NUMBER OF IMMUNE SPLEEN CELLS USED, PERCENT PLATED IN THE CONVENTIONAL MANNER, AND THE SELECTION METHOD UTILIZED ON THE REMAINING BULK CULTURE, FOR EACH OF THE 15 FUSIONS REPORTED IN THIS THESIS

FUSION NUMBER	TOTAL # OF CELLS USED ($X10^8$)	% PLATED IN CONVENTIONAL MANNER	SELECTION METHOD (WITH % OF BULK CULTURE USED)
1	1.7	70	I (100)
2	4.2	75	I (100)
3	3.5	85	I (100)
4	2.8	80	I (100)
5	4.2	75	I (70)*
6	1.5	75	P (100)
7	1.8	50	I (70) P (30)
8	4.3	90	P (100)
9	4.7	90	P (100)
10	3.1	80	I (70) P (30)
11	1.6	85	P (20)*
12	1.6	85	P (5)*
13	4.6	80	I (65) P (35)
14	0.78	0	I (100)
15	2.2	65	I (100)

I = Immunomagnetism
P = Panning
* The remainder of these bulk populations was discarded

2.1.5 MAINTENANCE OF HYBRIDOMAS

Both the 96-well plates and the 75 cm^2 flasks were incubated at 37°C, in a 10% CO_2 incubator, to allow the hybridomas to grow. After 48 hours, both were diluted 1:1 with 2x HAT selection medium. Following this, feeding was done every two to three days, by removing 50% of the culture medium and replacing it with 1x HAT medium.

The 96-well plates were treated in this way for a period of 10 to 15 days, until microscopic examination revealed that the majority of the hybridomas were ready for testing (when cell growth was approximately 50% confluent).

The bulk cultures were grown in their flasks for 4-7 days (pre-determined by kinetic studies; see Results Section). The hybridomas were then counted and subjected to Ag-specific selection using either panning or immunomagnetism. Following this, the total population of selected cells, plus a portion of the non-selected population, was plated in 96-well plates in the same manner as before. These plates were then incubated and fed in exactly the same way, until hybrid growth was determined to be sufficient for testing.

2.2 TESTING FOR SPECIFIC ANTIBODY PRODUCTION

All wells showing hybrid growth from the conventional

population, the selected population, and the non-selected population were screened for specific Ab production. Supernatant fluid (snf) from each well was tested against the immunogen in a cellular enzyme-linked immunosorbant assay (CELISA).

2.2.1 CELISA REAGENTS

i. PBS-TWEEN

-0.05% Tween-20 (BDH Chemicals) in PBS.

ii. BOVINE SERUM ALBUMIN (BSA)

-2 gm BSA (Sigma) were dissolved in 100 ml PBS to give a 2% BSA solution;

-this was diluted 1:4, with PBS, to give a 0.5% BSA solution.

iii. CONJUGATE (SECOND Ab)

Peroxidase-conjugated goat anti-mouse (GAM) IgG + IgM (Jackson ImmunoResearch) was diluted in 2% BSA, using a previously determined optimal dilution.

iv. PHOSPHATE CITRATE BUFFER

-0.1M Citric Acid: 1.92 gm anhydrous citric acid (Fisher Scientific) in 100 ml dH_2O;

-0.2M Phosphate: 2.82 gm dibasic, anhydrous sodium phosphate

(Sigma) in 100 ml dH_2O;

-4.9 ml of 0.1M citric acid was mixed with 5.1 ml of 0.2M phosphate. 10 ml dH_2O was added. The solution was mixed well and the pH adjusted to 5.0.

v. SUBSTRATE

-4 mg orthophenylenediamine (OPD) (Sigma) was dissolved in 10 ml phosphate citrate buffer;

-Immediately prior to use, 4 μl of 30% hydrogen peroxide (Sigma) was added.

vi. 2.5N SULPHURIC ACID

-to 93 ml dH_2O, 7 ml concentrated sulphuric acid (American Hospital Supply Canada Inc., Mississauga, Ontario) was added.

2.2.2 CELISA PROCEDURE USING ADHERENT CELLS

Since all fusions were done using mice immunized with transfectant cells, the primary screening procedure utilized the immunizing transfectant cell coated onto 96-well plates. The procedure was performed as follows:

-96-well, flat-bottomed tissue culture plates were coated with the immunizing transfectant cell, in 10% DMEM, by incubating overnight at 37°C, with a density of 5 x 10^4 cells per well;

-the following morning the wells were checked microscopically

to ensure confluent coating. Plates were then "flicked" into a waste receptacle to remove medium;

-plates were washed x1 by adding 100 μl PBS-Tween to the wells and flicking again;

-50 μl of snf from each fusion well showing hybrid growth was removed and added to a transfectant-coated well;

-plates were incubated for 1 hour at room temperature (RT);

-wells were flick/washed x4 with PBS-Tween, to remove unbound Ab;

-50 μl per well conjugate was added;

-plates were incubated 1 hour at RT;

-wells were flick/washed x5 with PBS-Tween, to remove unbound conjugate;

-100 μl per well substrate was added;

-plates were incubated 30 minutes, RT, in the dark;

-50 μl per well 2.5N H_2SO_4 was added to stop the color reaction;

-plates were read in a Multiscan Spectrophotometer (Bio-Rad model 3550 microplate reader) at 490 nm. Raw data reports were obtained.

2.2.2A SELECTION OF IMMUNOGEN-POSITIVE HYBRIDOMAS

For the CELISA assays, negative control wells (10% DMEM) and positive control wells (NFLD.M67 mAb: a mAb produced in this

laboratory which reacts with a monomorphic epitope on DP molecules) were included. Typically, negative control wells gave an optical density (OD) reading of approximately 0.05-0.20, while positive control wells gave an OD greater than 1.0. Positive fusion wells were then selected as any well giving an OD greater than 0.45.

Positive wells were identified and the corresponding hybridoma cells transferred to a 24-well plate (Nunc) to allow further growth. The positive supernatants were subsequently tested in a CELISA assay against a panel of target cells (transfectants and/or BCL), selected such that their HLA types would allow determination of the Ab specificities.

2.2.3 CELISA PROCEDURE USING NON-ADHERENT CELLS

CELISA using BCL as targets involves a modified procedure, as BCL do not adhere to tissue culture wells. The procedure used was performed as follows (note: both transfectant cells and BCL can be used in the following procedure, whereas the CELISA described above (section 2.2.2) is applicable to transfectants only):

-target cells were counted and the appropriate number centrifuged and washed x1 in 0.5% BSA (transfectant cells were used at a density of $5x10^4$ cells per well; BCL at $2.5x10^4$ cells per well);

-the cells were resuspended in 0.5% BSA and 10 μl was added to each well of a flexible, 96-well, poly-vinyl chloride, U-bottomed microtiter plate (Fisher Scientific);

-10 μl of snf from the positive hybridomas was added to each of two wells (duplicates) per target cell;

-plates were incubated for 1 hour at RT;

-wells were washed x3, by adding 100 μl per well of 0.5% BSA, centrifuging the plates at 300 x *g* for 5 minutes, flicking off the wash solution, and resuspending the cells;

-50 μl per well of conjugate was added;

-plates were incubated for 1 hour at RT;

-wells were washed x3 as before;

-cells were resuspended, and the flexible plates were "snap-fitted" onto Linbro/Titertek 96-well microtitration plates (ICN Biomedicals Canada, Mississauga, Ontario). 100 μl PBS was added to each well, and an 18-gauge needle was used to punch holes in the center of each well of the flexible plates, to allow cells to spin through into the Linbro plates;

-these plates were then centrifuged at 300 x *g* for 5 minutes;

-flexible plates were removed and discarded. Wash solution was flicked off the Linbro plates, and 100 μl per well substrate was added;

-plates were incubated for 30 minutes in the dark;

-50 μl per well H_2SO_4 was added;

-plates were then read as before. Positive control reports

were obtained.

2.2.3A SELECTION OF DP-POSITIVE HYBRIDOMAS

In the specificity screen described above, positives were defined as wells giving OD readings greater than 20% of the positive control (NFLD.M67). Results were tabulated and analyzed to identify DP-specific hybridomas. All hybridomas that appeared to be secreting mAb specific for DP were cloned, and the clones subsequently tested extensively to determine the specificity of each mAb. However, for the purpose of this thesis, only the primary and secondary screens are pertinant. The yield of Ag-specific Ab obtained after these initial screenings was the criterion used to determine whether the selection techniques were successful or not.

2.3 AG-SPECIFIC SELECTION USING PANNING

Ag-specific selection of post-fusion hybridomas using panning was attempted in eight fusions (see Table 4).

2.3.1 PANNING PROCEDURE

A method was devised to test this procedure, as follows:
-96-well flat-bottomed tissue culture plates were coated with

the immunizing transfectant cell, in 10% DMEM, by incubating overnight at 37°C, with a density of $5x10^4$ cells per well;

-the next morning, the wells were checked to ensure confluent coating, and the plates were then irradiated, using 1200 rads, to stop the transfectants from multiplying;

-following irradiation, the plates were flick/washed x2, with 10% DMEM, to remove unbound cells;

-the bulk culture of growing hybridomas was spun down, counted, and washed x3 at 4°C, using ice-cold 10% DMEM. This was done in order to minimize Ab secretion, as free Ab could interfere with the procedure;

-the hybridomas were resuspended in 10% DMEM and added to the transfectant-coated wells. Varying numbers of hybridomas per well were added in different experiments, in an attempt to optimize the conditions. This will be discussed in the Results section;

-the plates were incubated at 37°C for approximately 40 minutes;

-the plates were flick/washed x4, with 100 μl/well 10% DMEM;

-after the last wash, 100 μl of hybridoma medium containing 1x HAT was added to the wells, and the plates were incubated at 37°C to allow the bound hybridomas to grow;

-the unbound hybridomas (ie, those that had washed off) were collected, and a portion of this population was then plated in 96-well plates, using the same media and incubation conditions

as before;

-all plates were fed every 2-3 days with 1x HAT medium, until they were ready to test;

-once the hybridomas had begun to grow (usually about 5 days after the panning procedure), the "panned" plates were replicate plated in order to separate the growing hybridoma cells from the layer of transfectant cells. This was done to ensure that any specific Ab being secreted by the hybridomas would not be adsorbed by the transfectant cells, causing false negative results in the primary screen;

-all growing wells were tested for specific Ab production, using the CELISA technique, when it was determined that the hybrids had grown sufficiently (typically 10-15 days after plating).

2.4 AG-SPECIFIC SELECTION USING IMMUNOMAGNETISM

Ag-specific selection of post-fusion hybridomas using immunometallic beads was attempted in ten fusions (see Table 4). In the first eight, commercially available beads coated with GAM IgG (Dynal) were used. In these experiments, therefore, selection was based on IgG expression, rather than specificity for Ag. It was thought that this procedure, in itself, would increase the yield of Ag-specific hybridomas, since the desired memory cells should be producing antibody of

the IgG isotype. For the last two fusions, DP-coated beads were made and used for selection of Ag-specific hybridomas. Fusion experiments using GAM IgG-coated beads to select hybridomas had an additional step after testing for specific Ab. Since selection here was based on IgG expression, the optimal way of determining if the procedure had worked was to isotype the Ab being produced by hybridomas in the magnet-bound and non-bound populations; magnet-bound cells should be producing IgG Ab and non-bound cells should not. Therefore, in these eight fusions, isotyping was done on supernatant fluid from a random selection of wells showing hybridoma growth from both populations.

2.4.1 EXPERIMENTS TO OPTIMIZE USE OF GAM IgG-COATED BEADS

A preliminary set of experiments was performed, using GAM IgG-coated beads, in order to optimize the experimental conditions for selection of specific cells.

2.4.1A REAGENTS FOR OPTIMIZATION EXPERIMENTS

i. A20 cell line (American Type Culture Collection)
This is a mouse lymphoma line which expresses a high level of surface IgG (Kim et al, 1979; Glimcher et al, 1982), and thus provides an ideal prototype for optimizing these experimental

conditions. This cell line was cultured in 10% DMEM medium.

ii. SP2/O cells

These were included as a negative control, in order to assess the level of non-specific binding of beads.

iii. Carboxyfluorescein Diacetate (CFDA) (Sigma)

A stock solution was made by dissolving 10 mg in 1 ml acetone. This was stored at -70°C. A working dilution of 40 μl stock added to 5 ml PBS was used for cell staining. CFDA is a non-fluorescent compound which readily crosses intact cell membranes. Once inside, it is hydrolyzed by enzymes of the cell to produce a fluorescent compound.

2.4.1B PROCEDURES FOR OPTIMIZATION EXPERIMENTS

i. Cell staining using CFDA

In one experiment A20 cells and SP2/O cells were mixed together, and the beads were used to select the A20 cells from this mixed population. For this experiment, the A20 cells were prestained with CFDA in order to make them fluorescent, and thus distinguishable from SP2/O cells:

-3 x 10^6 A20 cells were washed x2 with PBS;

-200 μl of the working dilution of CFDA was added to the cell pellet;

-this suspension was incubated at 37°C for 20 minutes;
-the cells were washed x3 in PBS, followed by resuspension in 10% DMEM.

ii. Selection of A20 cells using GAM IgG-coated beads:
These preliminary experiments all followed the same basic procedure, as follows:
-the appropriate number of A20 cells (stained or unstained) and/or SP2/O cells was washed x2 in 10% DMEM;
-the appropriate number of GAM IgG-coated beads was added to a 15 ml centrifuge tube and washed x2 in FBS/PBS using the magnet/washing procedure;
-cells were added to beads, in 1-2 ml 10% DMEM;
-the suspension was incubated, while maintaining a gentle rocking motion of the tube;
-in some experiments, samples were removed at timed intervals and studied microscopically to determine the number of rosetted cells (a rosette was defined as any cell with two or more beads attached);
-to separate this suspension into magnet-bound and non-bound populations, 5 ml of 10% DMEM was added and the tube was placed in the magnet for 1-2 minutes. The supernatant was poured off into a 50 ml centrifuge tube (non-bound population), and the magnet-bound population was washed twice more, using 7 ml 10% DMEM each time. The supernatant was

poured off into the same 50 ml tube, which was centrifuged following the last wash. The magnet-bound and non-bound populations were then resuspended in small volumes of 10% DMEM and counted.

In these preliminary experiments, different bead:cell ratios and different incubation times and temperatures were tested, in order to find the optimal conditions. Once these had been established, immunometallic beads were used to select specific hybridomas from post-fusion populations.

2.4.2 SELECTION OF POST-FUSION HYBRIDOMAS USING IMMUNOMETALLIC BEADS

Selection of post-fusion hybridomas, using either GAM IgG-coated beads or DP-coated beads, was done as follows:

-the bulk cultures of growing hybridomas were centrifuged, counted, and washed x3 at 4°C, using ice-cold 10% DMEM;

-the appropriate number of beads was washed x2 in FBS/PBS, using the magnet/washing procedure; 10 beads per cell were used;

-beads were added to the hybridomas, in 15 ml centrifuge tubes, in 1-2 ml 10% DMEM;

-the suspension was incubated at 37°C, maintaining a gentle rocking motion, for approximately 30 minutes;

-the suspension was separated into magnet-bound and non-bound populations, using the magnet/washing procedure;
-the magnet-bound and non-bound populations were counted.
-the total population of magnet-bound cells, as well as a portion of the non-bound population, was plated in 96-well plates, at approximately 10 cells per well. Hybridoma medium with 1x HAT was the culture medium.
-these plates were incubated and fed as described previously, until they were ready to test. All growing wells were then tested for specific Ab production, using the CELISA technique.

2.4.3 ISOTYPING

2.4.3A REAGENTS FOR ISOTYPING

i. MOUSE SUB-ISOTYPING KIT
-(Bio-Rad, Mississauga, Ontario)

ii. BICARBONATE BUFFER
-1.59 gm sodium carbonate (Na_2CO_3) (Fisher Scientific) and 2.93 gm sodium bicarbonate ($NaHCO_3$) (Fisher Scientific) were dissolved in 1 liter dH_2O.

iii. CAPTURE AB
Affinipure GAM IgG + IgM (Jackson ImmunoResearch).

2.4.3B ISOTYPING PROCEDURE

-96-well Linbro plates were coated with capture Ab, diluted in bicarbonate buffer to a previously determined optimal dilution; 100 μl per well was added;

-plates were incubated at 4°C overnight;

-the next morning, plates were flick-washed x2 with PBS;

-to prevent non-specific binding, wells were filled with 300 μl 1% BSA/PBS;

-plates were incubated for 30 minutes at RT;

-plates were flick-washed x3 with PBS-Tween;

-100 μl of snf was added to each of 5 capture Ab-coated wells. Negative controls (10% DMEM) and positive controls (snf from hybridomas of known isotypes) were included;

-plates were incubated for 1 hour at RT;

-plates were flick-washed x4 with PBS-Tween;

-100 μl per well of rabbit anti-mouse (RAM) isotype specific panel reagents (in kit) were added; RAM IgG1 to first well; RAM IgG2a to second well; RAM IgG2b to third well; RAM IgG3 to fourth well; and RAM IgM to last well;

-plates were incubated 1 hour at RT;

-plates were flick-washed x5 with PBS-Tween;

-goat anti-rabbit horseradish peroxidase conjugate (from kit) was added. The conjugate was diluted 1/3000 with PBS-Tween, and 100 μl per well was added;

-plates were incubated 1 hour at RT;
-plates were flick-washed x4 with PBS-Tween;
-100 μl per well peroxidase substrate (from kit) was added;
-plates were incubated for 30 minutes at RT;
-plates were read in a Multiscan Spectrophotometer at 415 nm;
-positives were defined as wells giving an OD greater than 3x the negative control value.

2.4.4 DP-COATING OF IMMUNOMETALLIC BEADS

Immunometallic beads were coated with DP molecules according to the methods outlined in Chong Qing Qi's Masters thesis (Qi, 1993). Basically, beads coated with GAM IgG were first incubated with an anti-DP mAb, followed by incubation with a lysate made from a BCL expressing the same DP type as the immunizing transfectant cell.

2.4.4A COATING METALLIC BEADS WITH ANTI-DP ANTIBODY

2.4.4A.a REAGENTS

i. NFLD.M58
A mAb produced in this laboratory with specificity for an HLA-DP polymorphic epitope. Specificity analysis indicates the epitope to be DPβ1:55-56:DE.

ii. NFLD.M67

A mAb produced in this laboratory with specificity for an HLA-DP monomorphic epitope.

Both antibodies were used as hybridoma culture supernatants, obtained by overgrowing the hybridoma cells in 75 cm^2 tissue culture flasks, in 10% DMEM, for 3-4 weeks, followed by harvesting of the supernatant fluid. An assay done at the time of harvesting determined the concentration of NFLD.M58 to be 45 μg/ml and that of NFLD.M67 to be 115 μg/ml.

2.4.4A.b PROCEDURE

-approximately $20x10^6$ GAM IgG-coated beads were added to a 15 ml centrifuge tube and washed x1 in 1% FBS/PBS using a magnet;
-0.5 ml NFLD.M67 or 1 ml NFLD.M58 was added to the beads;
-this suspension was made up to 5 ml with 10% DMEM and rotated overnight at 4°C;
-the magnet was applied and the supernatant fluid was poured off;
-the beads were washed x4 by resuspension in 7 ml 1% FBS/PBS, followed by rotation for 30 minutes at 4°C;
-the beads were resuspended in 1% FCS/PBS;
-beads were checked for anti-DP coating using flow cytometry (Results Section).

2.4.4B PREPARATION OF CELL LYSATES

2.4.4B.a REAGENTS

i. Human B cell lines

MT14B (DPβ1:0402) and Jestholm (DPβ1:0401) were the two BCL used in these experiments (Yang et al, 1989).

These cells were grown in RPMI-1640 media (Gibco BRL) supplemented with 10% FBS, 2mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2mM sodium pyruvate (Gibco BRL).

The cells were grown in 75 cm^2 tissue culture flasks, at 37°C, in 5% CO_2, at a density not exceeding $8x10^5$/ml.

ii. 100mM stock phenylmethylsulfonyl fluoride (PMSF)

0.174 g PMSF dissolved in 10 ml ethanol. This stock solution was stored at -20°C.

iii. Lysing Buffer

-0.1576 g Tris-HCL was dissolved in 80 ml dH_2O and buffered to pH 8.2 with 5N NaOH;

-0.876 g NaCl, 37.22 mg EDTA, 100 mg BSA and 500 μl Nonidet P-40 were dissolved in the solution, which was then made up to 98 ml with dH_2O;

-this solution was stored at 4°C;

-immediately prior to use, 1/50 volume of stock PMSF was added;
-pH was measured and adjusted to 8.2 if required.

2.4.4B.b PROCEDURE

Cell lysates were prepared as previously described (Qi, 1993). Basically:
-the appropriate number of cells was washed x2 in ice-cold 10% RPMI in a 50 ml centrifuge tube;
-the cell button was resuspended in ice-cold lysing buffer - for every $1x10^8$ cells, approximately 3 ml lysing buffer was used;
-the suspension was mixed well and incubated at 4°C for approximately 20 minutes;
-the suspension was centrifuged for 30 minutes at 3500 rpm to sediment nuclei and debris;
-the supernatant was decanted and ready for use.

2.4.4C ADSORBTION OF DP MOLECULES BY Ab-COATED BEADS

-the appropriate number of metallic beads coated with anti-DP Ab was added to a 15 ml centrifuge tube and washed x1 with 1% FBS/PBS, using a magnet;
-the cell lysate solution was added to the beads and the tube

was rotated at 4°C overnight;

-the magnet was applied for 1-2 minutes and the supernatant was poured off;

-the beads were resuspended in 5 ml lysing buffer and rotated for 30 minutes at 4°C;

-the magnet was reapplied and the supernatant was again poured off;

-the beads were washed x3 by resuspension in 7 ml 1% FBS/PBS, rotating at 4°C for 30 minutes, and applying the magnet;

-the beads were resuspended in 1% FBS/PBS.

2.4.4D CHECKING COATED BEADS FOR DP EXPRESSION

In order to detect DP molecules on the beads, I3 conjugated to FITC (Coulter Immunology, Hialeah, FL) was used. I3 is a mAb specific for a monomorphic determinant on MHC class II molecules. Previous testing in this laboratory, using transfectant cells as targets, strongly indicated that this mAb was specific for HLA-DP with very little binding to DQ or DR. Cross-blocking experiments demonstrated that I3 blocked binding by NFLD.M58, but not by NFLD.M67 (Qi, 1993).

I3 is a mouse IgG Ab, and the metallic beads used here are coated with a goat ploYclonal antibody against all mouse IgG subclasses. Therefcre, I3 will bind non-specifically to unoccupied binding sites on the goat immunoglobulins. To

overcome this problem, the beads were first incubated with normal mouse serum (NMS), which was shown to be effective for blocking nonspecific fluorescence (Qi, 1993).

The staining was performed as follows:

-5 x 10^5 DP-coated beads were added to a 15 ml centrifuge tube;

-the magnet was applied in order to remove fluid;

-25 μl NMS was added and the tube was incubated at 4°C for 30 minutes;

-beads were washed x2 in azide buffer, using the magnet;

-10 μl FITC-I3 was added and the tube was again incubated at 4°C for 30 minutes;

-beads were washed x2 in azide buffer, as before;

-beads were resuspended in 200 μl paraformaldehyde and analyzed by flow cytometry.

Following this testing, the coated beads were ready for use in experiments designed to select DP-specific hybridomas from post-fusion cell populations, as described in section 2.4.2.

3.0 RESULTS

3.1 STUDY OF KINETICS OF POST-FUSION BULK CULTURES

Before attempting selection of Ag-specific hybridomas, an experiment was performed to study the growth kinetics of post-fusion populations cultured in flasks rather than in 96-well plates. This was considered necessary for several reasons:

(a) to ensure hybrid viability in bulk cultures.

(b) to determine the optimal number of post-fusion cells to culture in each flask

(c) to determine the optimal time to attempt selection. It was thought that selection should be attempted when the HAT selective medium had killed off all unfused SP2/O cells and the hybrids had stabilized and started to multiply.

(d) to determine the optimal time for addition of selective HAT medium. Conventional fusions in our laboratory always have HAT added on day 2, but it was thought that a faster SP2/O kill might prove advantageous for selection experiments. Therefore it was decided to compare HAT addition on day 1 versus day 2.

This experiment was first performed using a post-fusion population from a Balb/c mouse immunized with the BCL SLE-003 (Yang et al, 1989). The post-fusion population was cultured in six 75 cm^2 flasks, each containing 25 ml of hybridoma medium.

Three different concentrations of cells were cultured in each of two flasks, as follows:

-Flasks 1 and 2 contained 5 x 10^7 immune spleen cells (and therefore 5 x 10^6 SP2/O cells).

-Flasks 3 and 4 contained 2.5 x 10^7 immune spleen cells (and therefore 2.5 x 10^6 SP2/O cells).

-Flasks 5 and 6 contained 1.25 x 10^7 immune spleen cells (and therefore 1.25 x 10^6 SP2/O cells).

Flasks 1, 3, and 5 were fed with 25 ml 2x HAT on day 1, and flasks 2, 4, and 6 on day 2.

Small samples were removed daily from each flask and counted, for a period of 8 days. The SP2/O cells and the hybridomas were counted together, as they are not distinguishable by simple light microscopy; the smaller unfused spleen cells were ignored. The time of the lowest viable cell count was taken as the time of complete SP2/O death. An increasing count thereafter was interpreted as being due to hybrid growth.

Flask 3 became contaminated shortly after culture, and was therefore discarded, leaving 5 flasks for study.

Results of this experiment are shown in Figure 2.

It can be seen that all flasks showed slightly decreased counts on day 1. Flasks 1 and 5 (dotted lines), which were fed with HAT medium on day 1, had lower cell numbers again on day 2, while the other three flasks (solid lines), which had not been exposed to HAT medium, showed increasing counts. By day

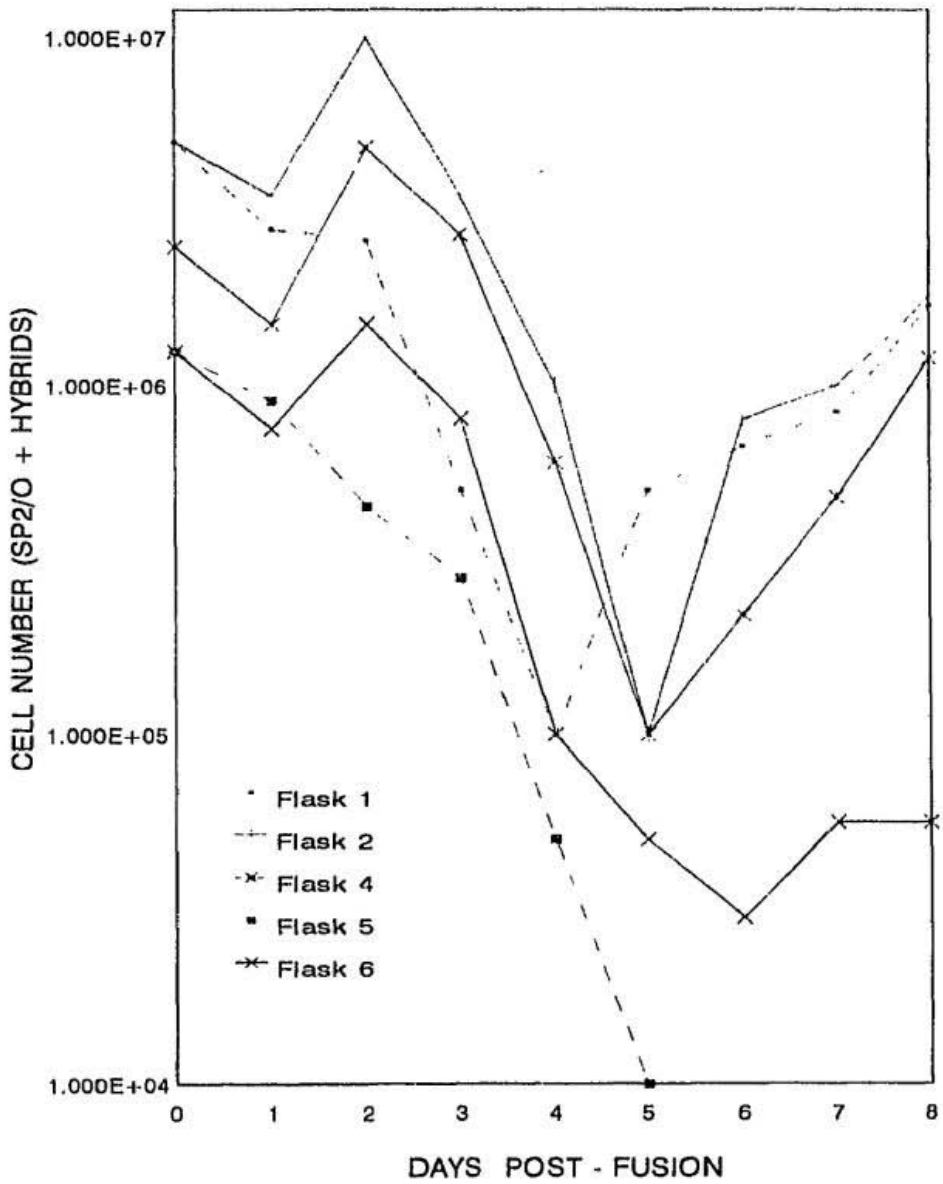


Figure 2. Growth curves for bulk populations of post-fusion cells from a BCL immunized mouse. Three concentrations of cells were tested (shown on the y axis at day 0), as well as initiation of HAT selection on day 1 (dotted lines) versus day 2 (solid lines).

3, when all flasks had been fed with HAT medium, decreasing cell numbers were apparent in all.

Flasks 1, 2, and 4 all had their lowest cell count three days after adding HAT medium. These three flasks also showed rapidly increasing counts following this low point, indicating that the hybrids were stabilized and growing successfully.

The two cultures containing the smallest number of cells, flasks 5 and 6, did not fare as well. Flask 5, which was fed with HAT on day 1, showed no viable cells after day 5. Flask 6, which received HAT on day 2, showed decreasing counts up to day 6. By the end of this experiment stable growth was still not established in this culture, although a small number of viable hybrids were still apparent. Therefore 1.25×10^7 immune spleen cells per flask was considered too low a number for successful growth of post-fusion bulk cultures.

Since this experiment was done on a post-fusion population from a mouse immunized with B cell line cells, it was decided to repeat the experiment on bulk cultures from transfectant immunized mice, to ensure that they followed a similar growth curve.

3.1.1 KINETICS OF POST-FUSION BULK CULTURES FROM TRANSFECTANT IMMUNIZED MICE

This experiment was repeated twice, using only one

concentration of cells (5 x 10^7 immune spleen cells per flask) each time, on post-fusion bulk cultures from C3H mice immunized with the transfectant DP8305. The growth curves for these two populations are shown in Figure 3. It can be seen that the two curves are very similar, and also very similar to the corresponding flask in the previous experiment (Flask 2). Therefore, bulk cultures from transfectant immunized mice were considered to follow the same kinetic pathway as those from BCL immunized mice.

From analysis of these results it was decided that

i) 2.5 x 10^7 immune spleen cells would be the lowest number cultured per flask. All subsequent post-fusion bulk cultures in this study contained 3.5 x 10^7 - 5 x 10^7 spleen cells.

ii) 2x HAT medium would be added on day 2. This was because the data from flasks 5 and 6 suggested that some hybrid cells may be killed by exposure to HAT medium within 24 hours of fusion, but may survive if exposure is delayed to 48 hours.

iii) selection of Ag-specific hybridomas would be attempted on bulk cultures growing for at least 5 days. At this point the SP2/O cells appeared to be dead, and the hybrids were healthy and proliferating.

3.2 SELECTION OF POST-FUSION HYBRIDOMAS USING IMMUNOMAGNETISM

Ag-coated metallic beads offered an attractive possible means

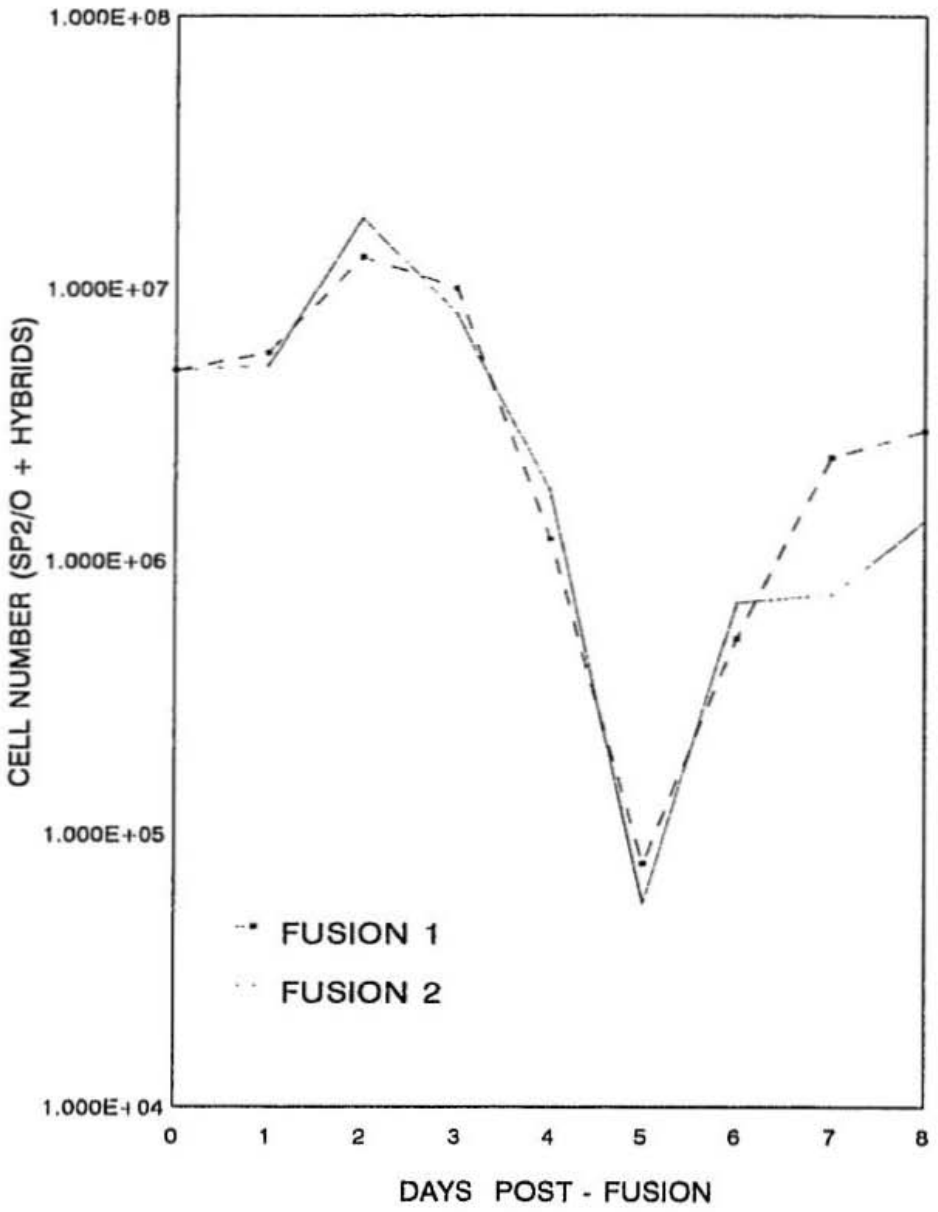


Figure 3. Growth curves for bulk populations of post-fusion cells from transfectant immunized mice.

for selecting Ag-specific hybridomas. In theory, any cell expressing Ig receptors specific for the Ag would bind these beads, forming rosettes. Application of a magnet would then allow rapid isolation of the rosetted cells from all other cells. Figure 4 gives a schematic diagram illustrating the principle behind this immunomagnetic procedure.

3.2.1 SELECTION EXPERIMENTS USING GAM IgG-COATED METALLIC BEADS

Before attempting to make DP-coated metallic beads, commercially available beads coated with goat anti-mouse (GAM) IgG (Dynal) were used for selection experiments on post-fusion populations from DP-transfectant immunized mice. It was theorized that this selection procedure, in itself, would increase the yield of Ag-specific hybridomas, as the desired cells should mostly be memory cells, and therefore of the IgG isotype.

In preliminary experiments, to establish optimal conditions, the cell line A20 was used. This is a mouse B cell lymphoma line which expresses a high level of surface IgG and thus offered an ideal model for optimizing experimental conditions using GAM IgG-coated beads.

A first set of experiments was done to ensure that the beads would indeed bind to the A20 cells. For this procedure, the

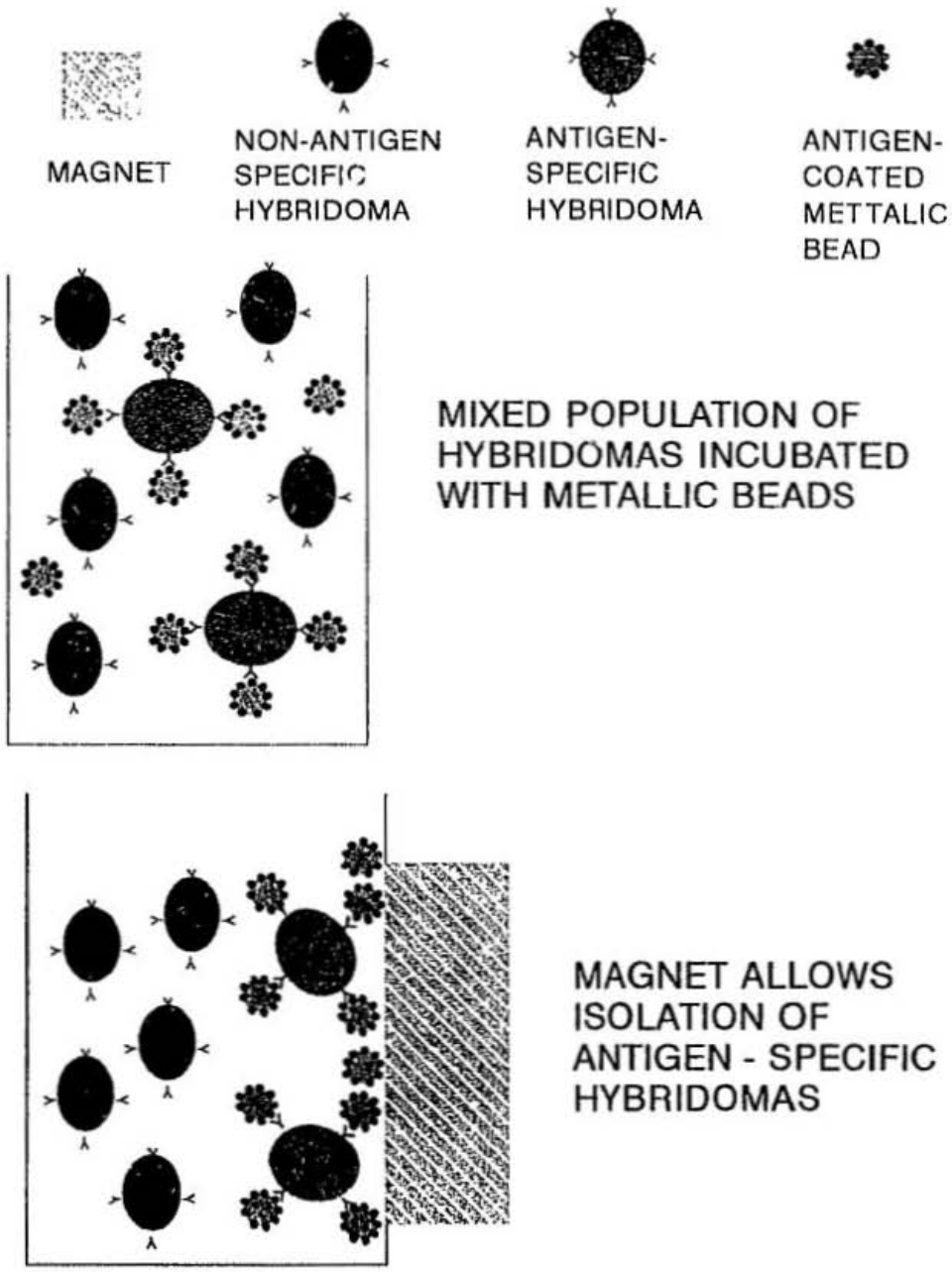


Figure 4. Schematic diagram illustrating the principle behind the immunomagnetic selection procedure used in this study.

experimental conditions of Ossendorp et al. (1988), whose experiments were similar to the ones reported here, were adopted. The procedure was performed as follows:

After washing and counting, beads and A20 cells were mixed together in 1-2 ml volumes, using 3 beads per cell. The suspension, kept at 4°C, was gently rocked for two hours, after which time the magnet was applied. Bound and unbound cell populations were then counted.

Using these conditions, it was consistently found that only about 50% of a population of A20 cells could be recovered in the bound population. Since these A20 cells express high levels of surface IgG (this was confirmed by a FACS analysis), it should have been possible to isolate close to 100% of these cells. Therefore it was decided that the experimental conditions used here were not adequate, and that optimal conditions needed to be established before proceeding.

3.2.1A OPTIMIZATION OF BEAD:CELL RATIO AND INCUBATION TIME AND TEMPERATURE

A. It was decided to test the bead:cell ratio first, as it was noticed that there were very few free beads in the magnet-bound populations from the experiments described above.

This set of experiments was performed as follows:

Cells and beads were counted, washed, and incubated together

in the same manner as before. Both A20 cells and SP2/O cells were tested (separately); SP2/O cells served as a negative control, to assess non-specific binding of the beads. 1 x 10^6 cells were used in each case, with bead:cell ratios of 3:1, 5:1, 10:1, and 20:1. Samples were removed at timed intervals and counted. Results, given in Table 5, are of rosetted and non-rosetted, rather than magnet-bound and non-bound, cells. Rosettes were defined as any cell with two or more beads attached.

From these results it was apparent that a bead:cell ratio higher than 3:1 was required. Ten beads per cell gave the best results, with an increase beyond this having no observed effect. It was therefore decided to use 10 beads per cell in all subsequent experiments. Non-specific binding of beads was negligible at all bead:cell ratios tested.

These results also showed that a one hour incubation time gave optimal results, with an increase beyond this having a detrimental effect. It was therefore decided to use an upper limit of one hour for incubations in subsequent experiments.

B. In the next set of experiments, summarized in Table 6, three incubation temperatures were tested. This experiment was performed in basically the same way as the previous one, except that ten beads per cell were used in each case. Incubation was done at 4°C, room temperature, and 37°C, and

TABLE 5: OPTIMIZATION OF BEAD:CELL RATIO AND INCUBATION TIME FOR IMMUNOMAGNETIC SEPARATION EXPERIMENTS

# of Beads per Cell	% Cells Rosetted at time (in minutes):							
	20		60		120		180	
	A20	SP2/O	A20	SP2/O	A20	SP2/O	A20	SP2/O
3	34	3	45	1	52	1	48	5
5	33	0	53	0	43	2	53	4
10	62	1	80	4	60	5	60	3
20	53	6	82	2	62	1	61	2

Incubation temperature was kept constant, at 4°C.

TABLE 6: OPTIMIZATION OF INCUBATION TIME AND TEMPERATURE FOR IMMUNOMAGNETIC EXPERIMENTS

Incubation Temperature	% cells rosetted at time (minutes)			
	30		60	
	A20	SP2/O	A20	SP2/O
4°C	47	1	57	1
Room temperature	90	0	92	2
37°C	97	2	98	3

The bead:cell ratio was kept constant, at 10:1.

samples were removed and counted after 30 minutes and 60 minutes.

It can be seen from these results that both room temperature and 37°C samples produced a much higher yield of rosetted cells than the 4°C sample. Since the 37°C sample showed the best results, it was decided to use this incubation temperature in subsequent experiments. Non-specific binding of beads was negligible at all three temperatures.

Table 6 also shows that, for room temperature and 37°C incubations, 30 minutes is an adequate incubation time, with an increase to 60 minutes having no apparent effect. Since incubating post-fusion hybridomas at 37°C could result in immunoglobulin synthesis and secretion, which would interfere with the procedure, it was desirable to use the shortest incubation time. Therefore a 30 minute incubation was used for subsequent experiments.

C. In a final set of experiments, the bead:cell ratio was tested again, using the newly established incubation conditions of 37°C for 30 minutes. As can be seen from the results in Table 7, ten beads per cell gave maximal rosetting under these incubation conditions as well. SP2/0 cells were not included here, as the previous two experiments had demonstrated that non-specific binding was negligible under all conditions tested.

TABLE 7: OPTIMIZATION OF BEAD:CELL RATIO FOR IMMUNOMAGNETIC EXPERIMENTS

# of Beads/cell	% cells rosetted	% cells in magnet-bound population
3	48	55
5	63	
10	93	85
20	97	

Incubation conditions were kept constant, at 37°C for 30 minutes.

This experiment included an additional step for two of the samples; in addition to counting the number of rosetted cells, these samples were separated into their magnet-bound and non-bound populations by the magnet/washing procedure. Both populations were counted, and Table 7 shows the percentage of cells present in the magnet-bound population. This was done to assess any detrimental effects of the washing procedure; if this process caused detachment of beads, the magnet-bound population would show a significantly lower percentage of cells than that obtained by simply counting the rosettes. As the results show, this was not found to be the case.

These experimental conditions - 10 beads per cell and incubation at 37°C for 30 minutes - were adopted for use in future experiments.

3.2.1B SELECTION OF A20 CELLS FROM A NON-IgG EXPRESSING POPULATION

Once these experimental conditions had been established, it was necessary to ensure that they worked on mixed populations of cells, allowing isolation of specific cells only (ie, IgG-expressing cells). It was decided to attempt selection of A20 cells, using GAM IgG-coated beads, from a mixed population of A20 and SP2/O cells.

For this experiment, in order to make the A20 cells distinguishable from the SP2/O cells, they were pre-treated with the dye carboxyfluorescein diacetate (CFDA). CFDA is a non-fluorescent compound which readily crosses intact cell membranes. Once inside, it is hydrolyzed by enzymes of the cell to produce a fluorescent compound. Thus, in this experiment, the A20 cells were fluorescent while the SP2/O cells were not, allowing analysis by flow cytometry.

The experiment was performed as follows:

The A20 cells were stained with CFDA as described in Materials and Methods section 2.4.1. Three cell mixtures were then prepared, each having a different ratio of SP2/O:A20 cells, as follows:

Sample #	% SP2/O cells	% A20 cells	SP2/O:A20 ratio
1	50	50	1:1
2	90	10	9:1
3	99	1	99:1

Each sample contained a total of 1×10^6 cells.

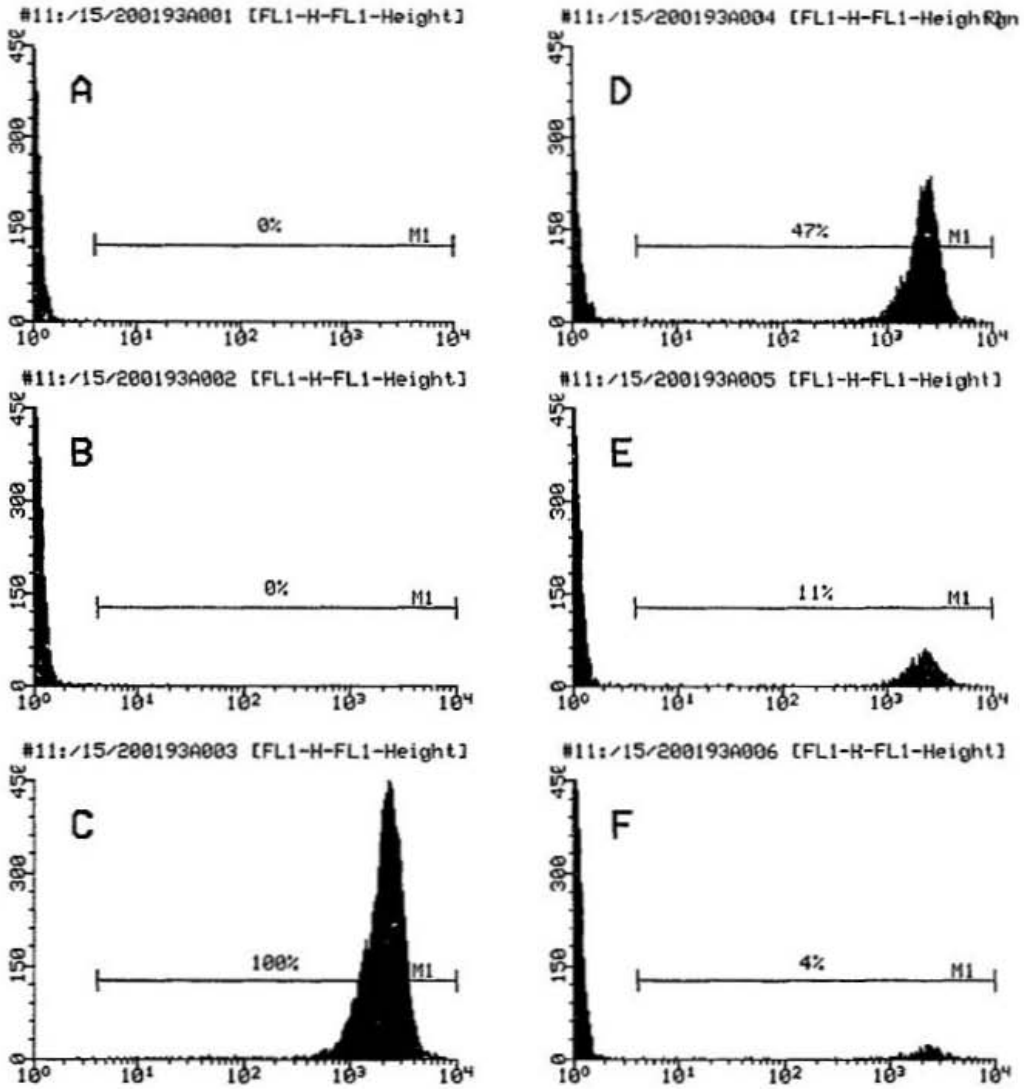
At this point, 1×10^5 cells from each sample were analyzed by flow cytometry. In addition, the same number of SP2/O cells, unstained A20 cells, and CFDA stained A20 cells were also analyzed. This was done to ensure that the staining had worked, and that the three samples contained approximately the right proportions of cells, before proceeding. Results are

shown in Figure 5a, panels A-F. Panels A and B show that there was no fluorescence in the SP2/O and A20 samples, while the CFDA stained A20 cells were highly fluorescent (panel C). Panels D, E, and F show samples 1, 2, and 3 respectively. It was apparent that each sample had approximately the correct SP2/O:A20 (ie, non-fluorescent:fluorescent) ratio, and therefore the experiment was continued.

1 x 10^7 GAM IgG-coated beads were added to each of the three mixtures, which were then incubated at 37°C for 30 minutes. The mixtures were separated into magnet-bound and non-bound populations using the magnet/washing procedure. The magnet-bound and non-bound populations were resuspended in 0.5 ml 10% DMEM and analyzed by flow cytometry.

These result are shown in Figure 5b, panels G-L.

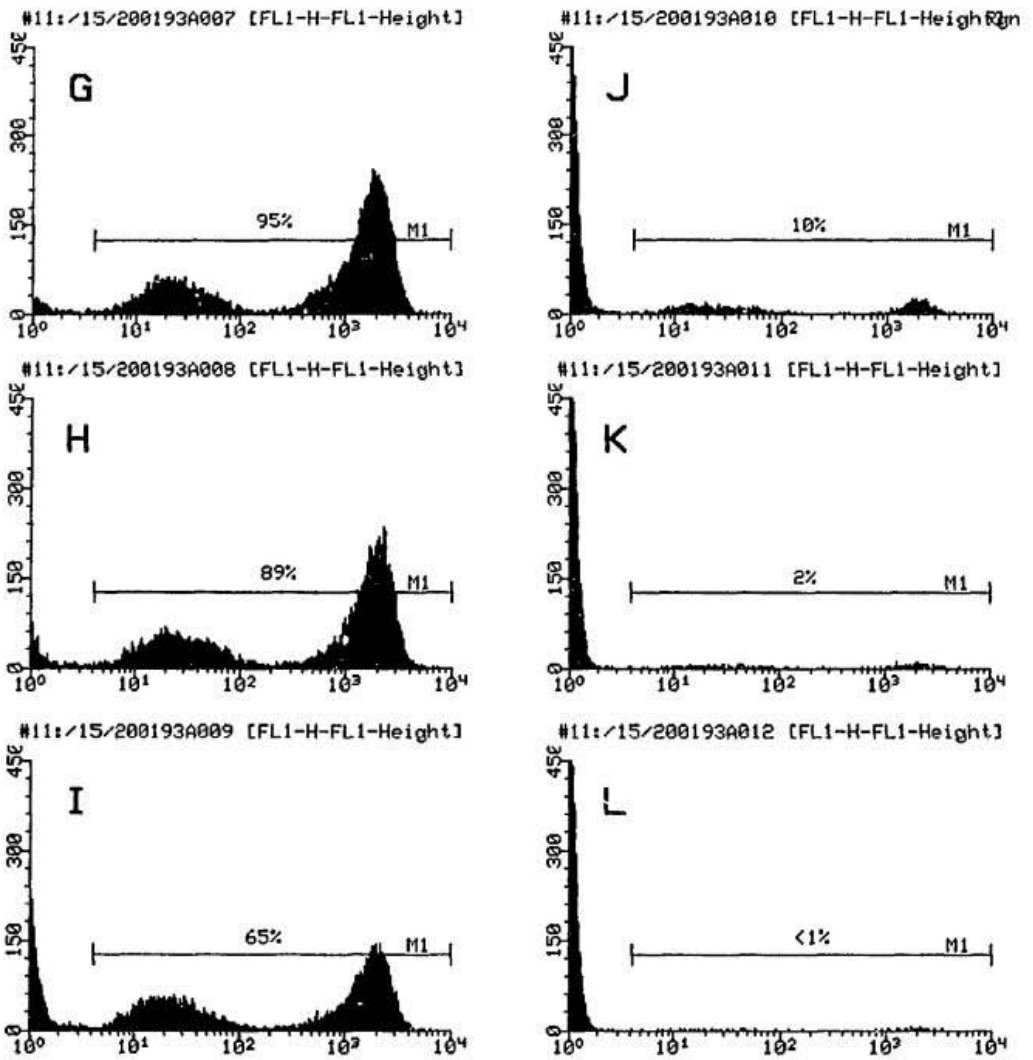
The first thing that was noticeable in these six histograms was a third population of cells, with a fluorescence intensity midway between that of SP2/O cells and stained A20 cells. Since this population was obviously positive for fluorescence, it was considered to represent stained A20 cells which had begun to "leak" CFDA. CFDA is retained by cells with intact membranes for a short period of time only. These samples were about 2 hours old at the time of analysis, and therefore it seemed reasonable that some cells would be losing their stain by this time. Panels A-F were analyzed immediately following cell staining, and did not show this third population.



A: SP2/O cells.
B: Unstained A20 cells.
C: CFDA-stained A20 cells.

D: Sample 1 (50% SP2/O; 50% A20).
E: Sample 2 (90% SP2/O; 10% A20).
F: Sample 3 (99% SP2/O; 1% A20).

Figure 5a. Flow cytometry results for experiment designed to test GAM IgG-coated immunometallic beads, using mixtures of SP2/O and fluorescent A20 cells.



G: Magnet-bound cells - sample 1.
H: Magnet-bound cells - sample 2.
I: Magnet-bound cells - sample 3.

J: Non-bound cells - sample 1.
K: Non-bound cells - sample 2.
L: Non-bound cells - sample 3.

Figure 5b. Flow cytometry results for experiment designed to test GAM IgG-coated immunometallic beads, using mixtures of SP2/O and fluorescent A20 cells.

Later experiments (not shown) confirm that stained but unmanipulated samples, such as those in panels C-F, also have this third population if left for the same period of time.

Panels G, H, and I represent the magnet-bound populations for samples 1, 2, and 3 respectively, with all three showing a large enrichment for fluorescent cells; sample 1, which had 47% fluorescence in the untreated sample, showed 95% fluorescence in the magnet-bound population. Similarly, sample 2 increased from 11% to 89%, and sample 3 from 4% to 65%. These results indicated that the beads were, indeed, selecting mostly A20 cells, with the purity of the selected population decreasing as the percentage of specific cells in the starting population decreased. Thus sample 3, with the lowest percentage of specific cells (4%) in the untreated population, showed the highest level of non-specific cell trapping (35%). However, since this sample contained only 1 x 10^4 A20 cells to begin with, this 35% represents a very small number, indicating that non-specific trapping does not present a major problem here.

Panels J, K, and L show the non-bound populations for samples 1, 2, and 3 respectively. In each case there was a very significant decrease in the number of fluorescent cells present, confirming that the beads had bound most of the A20 cells, thus depleting them from the non-bound populations. However, these histograms also show that the beads were not

isolating all the A20 cells. Sample 1, which had 47% fluorescence in the untreated sample, still had 10% fluorescence in the non-bound population. Similarly, sample 2 decreased from 11% to 2% and sample 3 from 4% to < 1%. Thus, in all cases, approximately 75% - 80% of the A20 cells were isolated by the immunomagnetic beads.

These results, though imperfect, were considered acceptable for the intended purpose. It was therefore decided to begin selection experiments on post-fusion hybridomas, using the established experimental conditions.

3.2.1C SELECTION OF POST-FUSION HYBRIDOMAS USING GAM IgG-COATED BEADS

A total of eight experiments were performed utilizing GAM IgG-coated metallic beads. The beads were used to select specific hybridomas from post-fusion bulk cultures obtained from mice immunized with DP transfectant cells. For each experiment, selection was attempted four to seven days post-fusion. The fusion details are described in Materials and Methods, particularly Tables 1, 3, and 4. The immunomagnetic procedure described in Materials and Methods section 2.4.1 was adhered to for these eight fusions (ie, 10 beads per cell and incubation at 37°C for 30 minutes).

The details of these selection experiments are listed in table

8. As this table shows, between one and ten percent of the total number of hybridomas were selected by the GAM IgG-coated beads in each experiment.

The magnet-bound population and a sample of the non-bound population were plated separately in 96-well plates. These plates were maintained as described previously until ready for testing. Table 9 gives the results of the primary screen - a CELISA assay using, as targets, the transfectant cell that had been used for immunization - for these eight experiments. For each fusion, the total number of wells showing hybrid growth (column A) and the number positive against the immunizing Ag on the primary screen (column B) are reported, for each of the three resulting populations; ie, the conventional population (plated immediately following fusion), the magnet-bound population, and the non-bound population.

It can be seen that, in each fusion, an increased yield of Ag-specific hybridomas was obtained in the magnet-bound population, as compared to the conventional population. The total numbers are summarized in the last row of this table. For the conventional population, 4% of the total number of growing wells were found to contain Ag-specific antibody. For the magnet-bound population, this was increased to 14%, indicating that the yield of Ag-specific cells was 3.5 times higher in this population. For the non-bound population, 2% were positive, indicating that Ag-specific cells were less

Table 8: DETAILS OF EXPERIMENTS UTILIZING GAM IgG-COATED IMMUNOMETALLIC BEADS TO SELECT POST-FUSION HYBRIDOMAS

Fusion Number	# Days post-fusion	Magnet-bound population	Non-bound population	% of total in magnet-bound pop.
1	7	3.0×10^3	5.0×10^4	6
2	5	4.0×10^3	1.0×10^5	4
3	5	5.0×10^3	6.0×10^4	8
4	6	5.0×10^4	6.0×10^5	8
5	7	8.4×10^3	8.2×10^5	1
7	5	1.7×10^3	1.1×10^5	2
10	4	8.4×10^3	1.0×10^5	8
13	4	7.2×10^3	6.5×10^4	10

TABLE 9: RESULTS OF PRIMARY SCREENING CELISA FOR FUSIONS UTILIZING GAM IgG-COATED IMMUNOMETALLIC BEADS TO SELECT POST-FUSION HYBRIDOMAS

Fusion Number	Conventional Population		Magnet-bound Population		Non-bound Population	
	A	B	A	B	A	B
1	800	16 (2)	480	95 (20)	480	11 (2)
2	1840	62 (4)	59	12 (20)	89	1 (1)
3	1680	49 (3)	21	5 (24)	31	1 (3)
4	1500	100 (7)	43	5 (12)	49	2 (4)
5	1690	16 (1)	600	35 (6)	252	6 (2)
7	192	12 (6)	50	15 (30)	ND	ND
10	1816	125 (7)	240	47 (20)	18	0 (0)
13	2160	70 (3)	159	11 (7)	30	0 (0)
TOTALS	10166	450 (4)	1652	225 (14)	949	21 (2)

Column A: Total number of wells with growing hybridomas.

Column B: Number (percent) of wells positive against the immunizing cell in the primary screening CELISA.

ND : Not done.

frequent, but not completely depleted, from this population. Since selection here was based on IgG expression rather than on Ag specificity, these results were considered acceptable.

3.2.1C.a ISOTYPING

Based on the nature of the selection method used in these experiments, it was expected that the magnet-bound population would contain more hybrids producing IgG. Thus IgG production could be taken as a measure of success of the method. A random selection of wells showing hybrid growth from both populations in each of these eight fusions was thus subjected to the isotyping procedure described in Materials and Methods 2.4.1C. For the first four of these eight experiments, very poor isotyping results were obtained. Virtually every well tested was positive for IgM, with some showing IgG as well. These results strongly indicated that the selection procedure was not working at all. However, since the primary screening results for these fusions (table 9) showed that selection had indeed increased the yield of Ag-specific hybrids, the poor isotyping results were contradictory. An effort was therefore made to explain these confusing results.

i. Trouble-shooting:

In an attempt to determine the reason for these conflicting

results, it was theorized that the feeder cells in the hybridoma medium used for cell culture might be secreting non-specific Ab which was being detected in the isotyping procedure. In order to test this theory, 10 rows of a 96-well plate were set up so that each row contained a different mixture as follows

ROW	CONTENTS OF WELLS	IgM
1	20% DMEM	-
2	20% DMEM + RBC	-
3	20% DMEM + irradiated spleen cells	+
4	20% DMEM + non-irradiated spleen cells	+
5	A20 cells + 20% DMEM	-
6	A20 cells + 20% DMEM + RBC	-
7	A20 cells + 20% DMEM + irrad spleen cells	+
8	A20 cells + 20% DMEM + non-irrad spleen cells	+
9	A20 cells + 20% DMEM + RBC + irrad spleens	+
10	A20 cells + 20%DMEM + RBC + non-irrad spleens	+

All wells contained a total volume of 100 μl. A20 cells were included in order to mimic hybridoma growth and were cultured at 10 cells per well. RBC and spleen cells were used at the same concentration as in regular hybridoma medium; ie, RBC at $2x10^7$/ml; spleen cells at $1x10^6$/ml. After a 48 hour incubation, 100 μl per well of 20% DMEM was added and the plate was incubated for another three days. Supernatant was removed from

all wells and tested for the presence of IgM using the isotyping procedure already described. IgM was the only isotype tested for, as most non-specific Ab will be IgM. Results of this experiment, shown in the third column of the table above, showed no IgM in any of the wells from rows 1, 2, 5, and 6. However, 100% of wells from rows 3, 4, 7, 8, 9, and 10 showed a high level of IgM. This confirmed that the feeder spleen cells, whether irradiated or not, were secreting IgM, which was being detected by the isotyping procedure. It was therefore decided that, for subsequent experiments of this nature, one or two 96-well plates from both the magnet-bound and the non-bound populations would be cultured using RBC only as the feeder cells. As was expected, these plates yielded fewer wells with growing hybridomas. However, growth was sufficient to enable selection of a random sample for isotyping purposes.

ii. Interpretation of isotyping results:

Table 10 gives the isotyping results for the four experiments that included plates cultured using only RBC as the feeder cells. These results indicated that the procedure was successful in enriching for IgG positive hybridomas in the selected population. The total magnet-bound population was found to have 75% IgG positive wells. Thus 25% of the hybridomas in this population were non-IgG secreting; these

Table 10: ISOTYPING RESULTS FOR EXPERIMENTS UTILIZING GAM IgG-COATED IMMUNOMETALLIC BEADS TO SELECT POST-FUSION HYBRIDOMAS

Fusion Number	Magnet-bound population		Non-bound population	
	# Isotyped	IgG +ve	# Isotyped	IgG +ve
5	26	17 (65%)	18	2 (11%)
7	18	14 (78%)	ND	ND
10	23	20 (87%)	20	7 (35%)
13	9	7 (78%)	8	0 (0%)
Totals	76	58 (76%)	46	9 (20%)

probably represent cells that were trapped, non-specifically, by the beads during the procedure. This non-specific binding was not thought to be a major problem, since the total number of hybridomas selected was still very small.

The non-bound populations, however, indicated a more serious problem. The total non-bound population contained 20% IgG positive wells. These probably represent IgG secreting hybridomas which escaped selection. There are several reasons why a cell might escape this selection method, with the most probable being that the cell is secreting, but not expressing, IgG. Another possibility is that the cell has undergone isotype switching, from IgM at the time of selection to IgG at the time of testing.

Regardless of the reason, the fact that the non-bound populations contained an average of 20% IgG positive wells was disappointing. It was hoped, initially, that this selection procedure would result in isolation of virtually all Ag-specific cells, and be useful for screening large post-fusion populations containing very small numbers of specific cells. This average of 20%, however, may be misleading. These experiments selected for IgG positive cells, and are therefore not directly comparable to Ag-specific selection, where the number of specific cells would be much lower. In addition, results for only three fusions are reported, with one giving 35% IgG positive wells, while the other two give 11% and 0%.

It would therefore be necessary to have additional data in order to make definite conclusions as to these results. Since it was evident that the beads were selecting mostly specific cells, and since time was a factor, we decided to proceed with the next set of experiments using DP-coated beads. It was hoped that the efficiency of the procedure would be increased by selecting Ag-specific, as opposed to IgG-expressing, hybridomas.

3.2.2 SELECTION EXPERIMENTS USING DP-COATED BEADS

Two selection experiments in this study were done using HLA-DP-coated beads. The beads were prepared as described in Materials and Methods section 2.4.4. Basically, beads coated with GAM IgG were first incubated with an anti-DP mAb, followed by incubation with a cell lysate prepared from a BCL expressing the same DP type as the immunizing transfectant cell. In theory, the anti-DP mAb would bind to the DP molecules present in the cell lysate, to give DP-coated beads. Figure 6 gives a schematic diagram of the steps involved in this procedure.

It was decided to use BCL, as opposed to the immunizing transfectant cell, as the source of DP molecules for coating beads, as experiments in our laboratory have indicated that BCL express significantly more DP than transfectant cells.

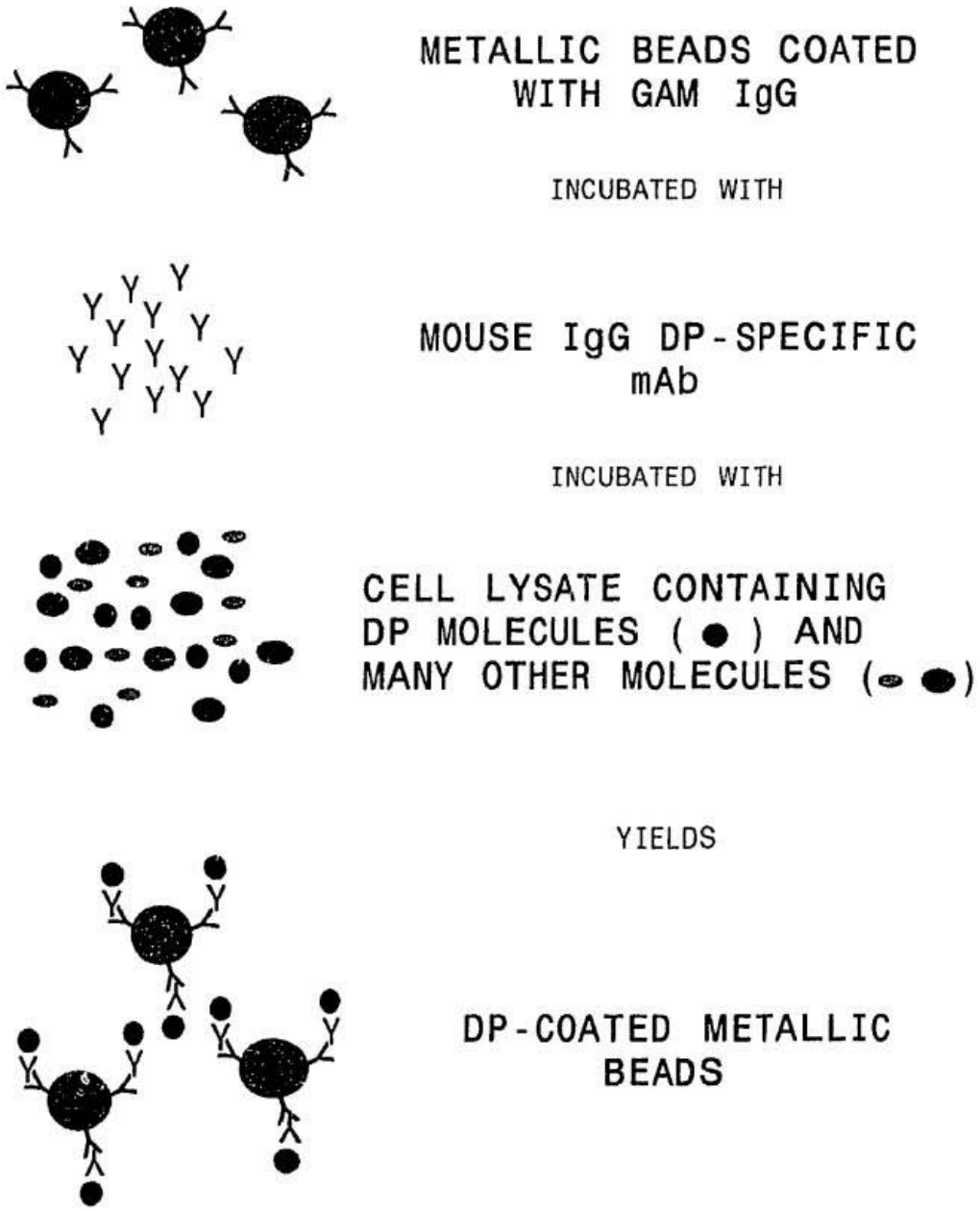


Figure 6. Schematic diagram illustrating the steps involved in the preparation of DP-coated beads, for use in immunomagnetic selection of Ag-specific hybridomas.

3.2.2A CHOICE OF ANTI-DP mAb FOR COATING METALLIC BEADS

For these experiments, it was desirable (but, as will be seen, only possible in the first experiment) to use two mAb, which bind to non-overlapping epitopes on the immunizing DP molecule, to coat the GAM IgG-coated metallic beads. The reason for this is that the epitope that binds to the anti-DP mAb becomes blocked, and thus the beads are rendered incapable of selecting hybridomas with the same specificity as the coating Ab. By using two Ab with non-overlapping binding sites, all epitopes are made available, and this problem is overcome.

NFLD.M58 and NFLD.M67 mAb, both produced in this laboratory, are two such antibodies. M58 binds to a polymorphic determinant on DP molecules (DPβ1:55-56:DE), while M67 binds to a monomorphic DP epitope. The two antibodies have previously been shown to not block each other (Qi, 1989). In addition, it has been shown that the fluorescent Ab I3, used in these experiments to detect DP molecules adsorbed onto metallic beads, is blocked by M58, but not by M67 (Qi, 1989).

3.2.2B COATING METALLIC BEADS WITH ANTI-DP mAb

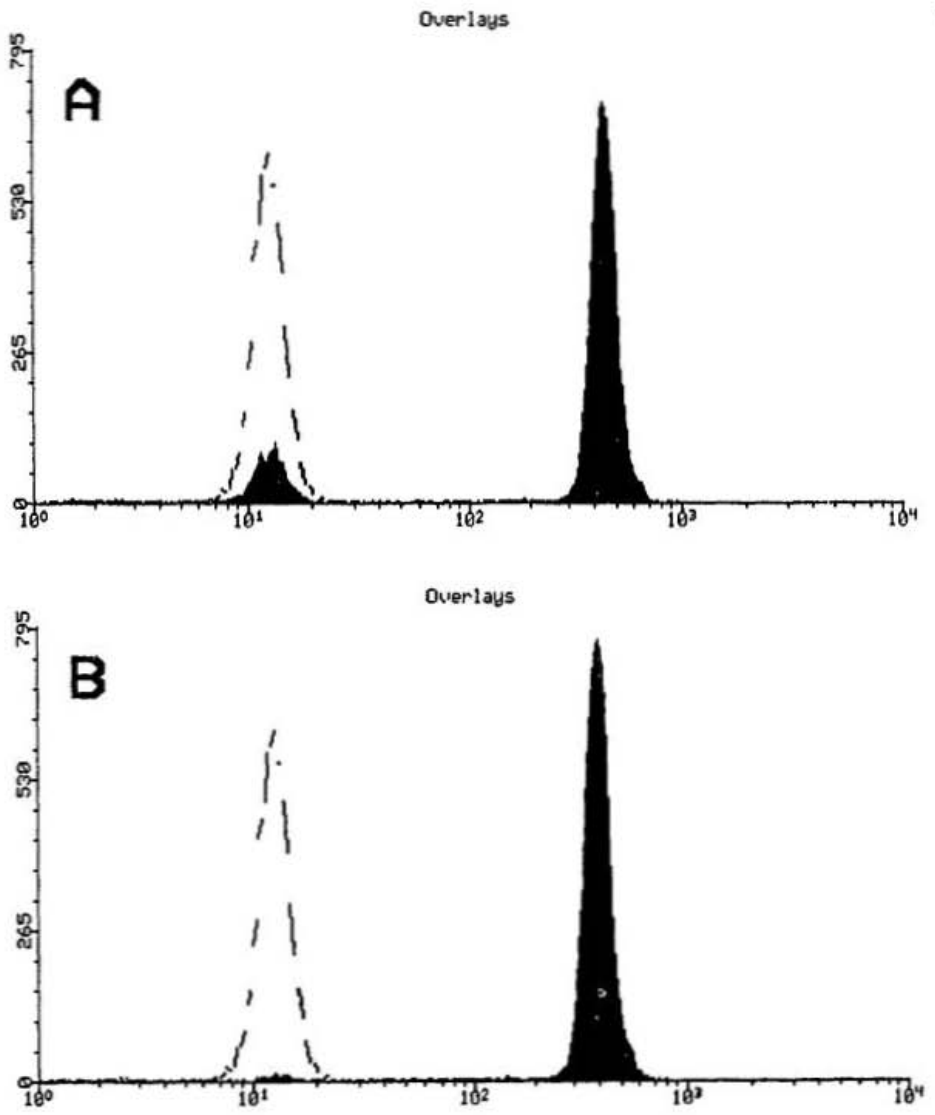
GAM IgG-coated beads were coated with NFLD.M58 and NFLD.M67 antibodies as described in Materials and Methods section

2.4.3. The concentrations used here had previously been determined to be optimal.

Following this procedure, the beads were stained with R-phycoerythrin-conjugated GAM IgG and analyzed by flow cytometry, to ensure that the beads were coated with mAb. GAM IgG-coated beads that had not been exposed to anti-DP mAb were used as a negative control. The results are shown in Figure 7. In each panel, the clear histogram represents the uncoated beads, while the solid histogram represents the anti-DP-coated beads. Panel A shows results for NFLD.M67-coated beads; panel B for NFLD.M58-coated beads. Both M58 and M67 coated beads show a high level of fluorescence, with the uncoated beads showing virtually none. Although a very small population of uncoated beads was apparent in the M67-coated sample, it was evident that the beads were indeed adequately coated with anti-DP mAb.

3.2.2C ADSORBTION OF DP MOLECULES FROM CELL LYSATES BY METALLIC BEADS

A preliminary experiment was done to determine the optimal ratio of the number of cells used for making the lysate to the number of beads used for coating (cell:bead ratio). It was thought that a ratio of approximately 2 cells for every bead would be sufficient to allow adequate DP coating. To test this



Panel A: NFLD.M67-coates beads.
Panel B: NFLD.M58-coated beads.
Clear histograms: Uncoated beads.
Solid histograms: Anti-DP-coated beads.

Figure 7. Flow cytometry results for detection of anti-DP mAb coated onto GAM IgG immunometallic beads.

an experiment was performed as follows:

-MT14B cells were used to make cell lysates, according to the procedure described in Materials and Methods section 2.4.4B. Four different lysates were prepared, using 2 x 10^6 cells, 4 x 10^6 cells, 8 x 10^6 cells, and 12 x 10^6 cells;

-half of each lysate was added to 1 x 10^6 M58 coated beads and the other half to 1 x 10^6 M67 coated beads; this gives cell:bead ratios of 1:1, 2:1, 4:1, and 6:1;

-following adsorbtions, the beads were stained with FITC-I3, as already described, to detect DP coating.

Results are shown in Figure 8. For each panel, the clear histogram represents the negative control (beads coated with anti-DP mAb only) while the solid histogram represents the DP-coated beads. (In panels E-H the clear histograms are not apparent because they are completely overlapped by the solid histograms).

Panels A, B, C, and D show results for M67-DP-coated beads with cell:bead ratios of 1:1, 2:1, 4:1, and 6:1 respectively. Panels E, F, G, and H show results for M58-DP-coated beads with the same cell:bead ratios.

It can be seen that the M67-DP-coated beads show a shift between the negative control and the test samples, while the M58-DP-coated beads show no shift at all.

This is the expected result, as it had previously been determined that M58 and I3 block each other, whereas M67 and

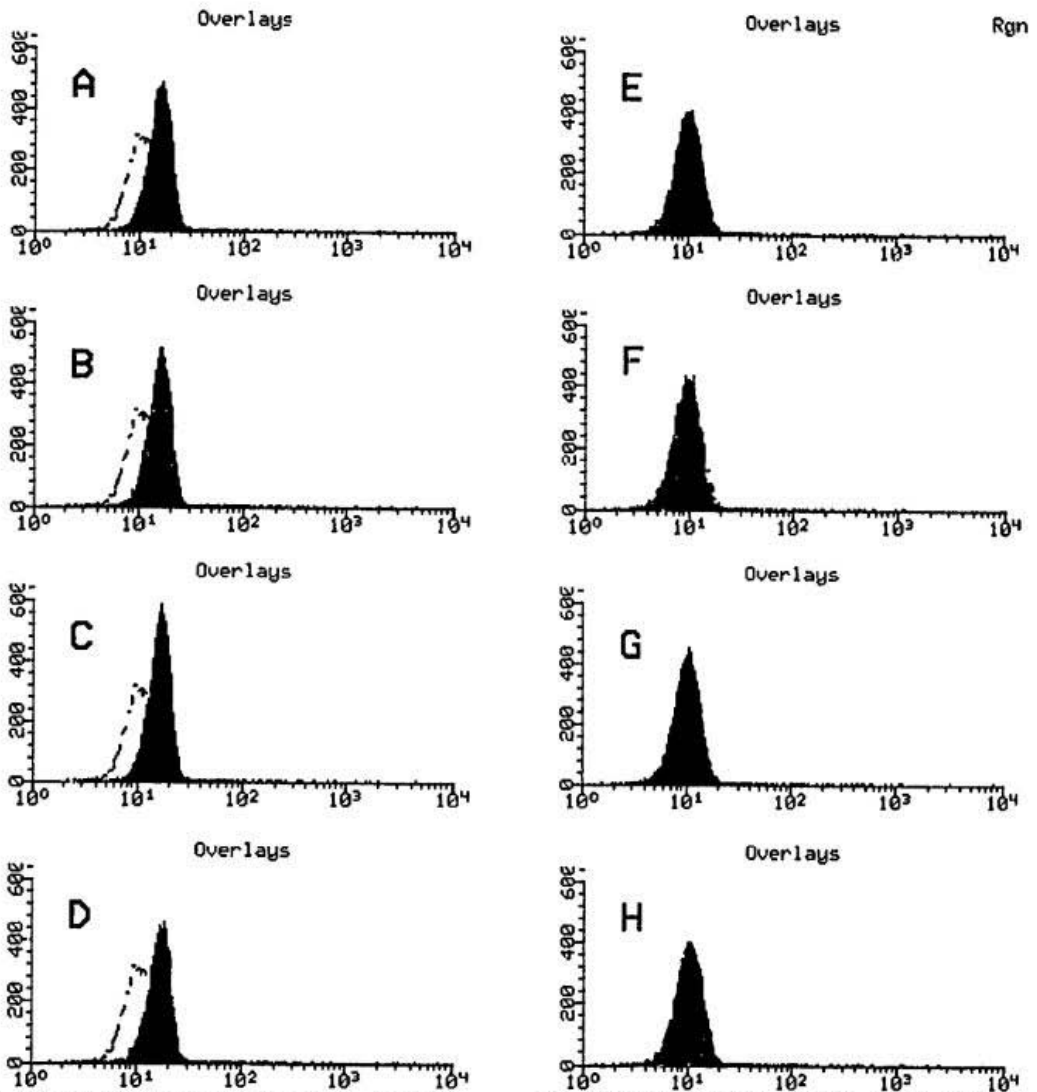


Figure 8. Flow cytometry results for detection of DP molecules, adsorbed from cell lysates, on anti-DP-coated immunometallic beads. Different numbers of cells were lysed and used for coating a constant number of beads, to determine the optimal cell:bead ratio.

I3 do not. This in itself can be taken as evidence that the beads have adsorbed DP molecules from the cell lysate. The shifts seen with the M67-DP-coated beads show that there is virtually no difference in the amount of DP adsorbed from any of the lysates. This indicates that saturating levels of DP molecules are present in the 1:1 ratio sample. For fusion numbers 14 and 15, reported below, a ratio of approximately 5:1 was used.

3.2.2D FUSION NUMBER 14

This fusion was done using spleen cells from a mouse immunized with the transfectant DP8305 (DPβ1:0402). The experiment was performed in the same manner as the others reported in this thesis, except that none of the fused cells were plated in the conventional manner - the total fused population was grown as a bulk culture. The selection experiment was done on day 5 post-fusion. At that time, 10% of the bulk culture was plated directly, using 10 cells per well (unmanipulated population). The other 90% was selected, using DP-coated beads.
Since NFLD.M58 and NFLD.M67 react with DPβ1:0402, both M58-coated and M67-coated beads were used (separately) for immune adsorbtion of DP molecules from lysates prepared using the BCL MT14B (DPβ1:0402). Following adsorbtion, the coated beads were stained with FITC-I3, and the results are shown in Figure 9.

From these results it can be seen that the M67-DP-coated beads (panel A) show a shift in fluorescence intensity as compared to the negative control, while the M58-DP-coated beads (panel B) show no shift at all. Again, because M58 and I3 block each other, this is the expected result and indicates that the beads have indeed adsorbed DP molecules from the cell lysate. These DP-coated beads were then used for the selection experiment, with ten of each, per cell, in the final selection mixture (ie, total of 20 beads per hybridoma cell).

Following selection, the magnet-bound and non-bound populations were counted:

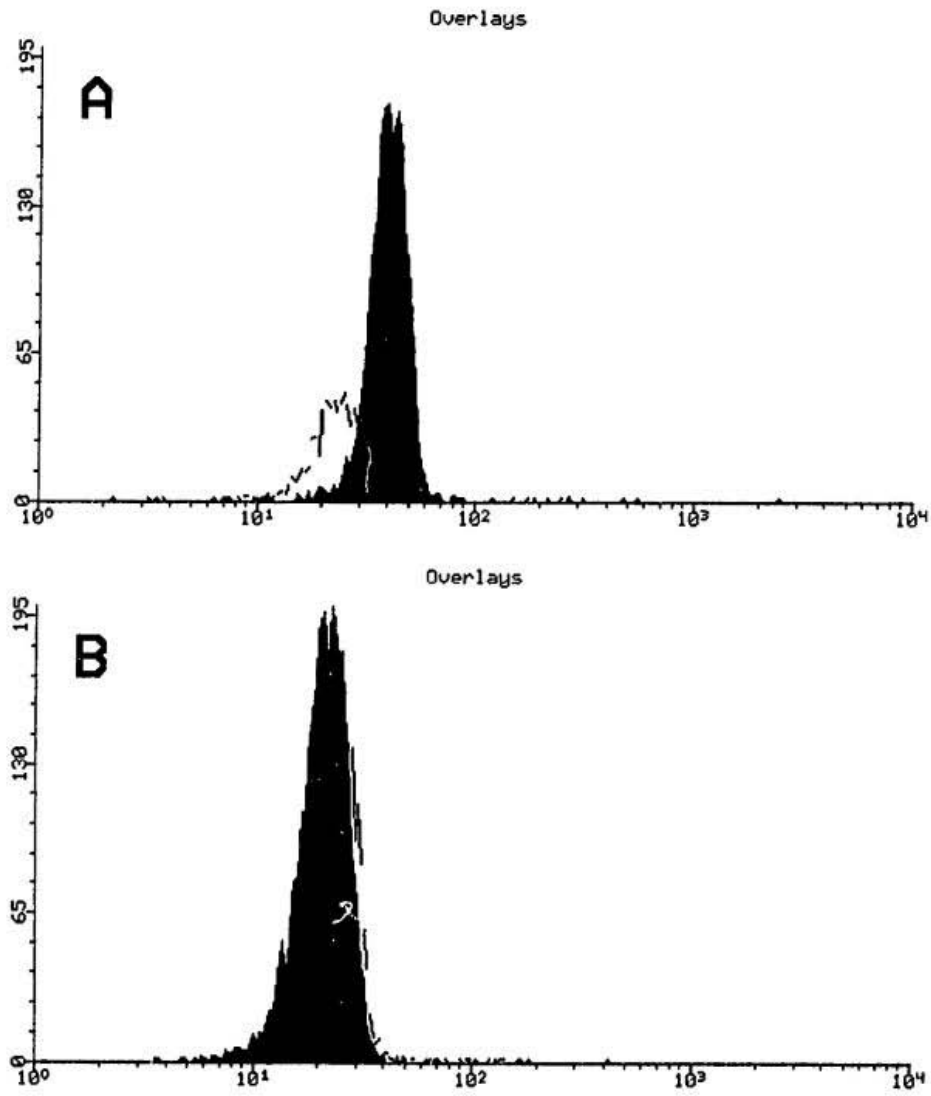
magnet-bound population: 6×10^3 hybridomas.

non-bound population: 8.5×10^4 hybridomas.

Therefore, 7% of the total number of hybrids were selected by the DP-coated beads.

The total population of magnet-bound cells was plated, using 10 cells per well. For the non-bound population, the intent was to plate the same number of cells (ie, 6×10^3), in the same manner. However, it was retrospectively discovered that a mistake had been made in the calculations, resulting in only 1/10 the desired number of cells (ie, 6×10^2) being plated. This explains the very low number of wells from this population that were found to contain growing hybridomas.

These plates were maintained as previously described, and all growing wells were tested for specific Ab production when



Clear histograms: uncoated beads.
Solid histograms: DP-coated beads.

Figure 9. Flow cytometry results for detection of DP molecules, adsorbed from an MT14B BCL lysate, on immunometallic beads coated with NFLD.M67 (panel A) and NFLD.M58 (panel B) anti-DP mAb.

growth was determined to be sufficient. Since selection was done using DP molecules from MT14B cells, the CELISA assay utilized these same cells as the target cells. The results of this CELISA are presented below:

	Bulk culture unmanipulated	Magnet-bound population	Non-bound population
Total # wells with growth	96	240	33
# and (%) +ve against MT14B	1 (1%)	15 (6%)	0 (0%)

On one hand these results look quite good; one positive hybridoma was found in 96 unmanipulated wells, so 2-3 positives would be expected from the 240 magnet-bound wells, if no selection had occurred. Since 15 positives were found, this gives an indication that the procedure has been at least partially successful.

On the other hand, only 6% of the magnet-bound population was Ag-specific, indicating a high degree of non-specific trapping by the beads. However, since the number of cells selected is still very low (240 growing wells in this case), this degree of non-specific binding does not pose a major problem. With the extremely low number of Ag-specific cells available, non-specific binding is almost a certainty.

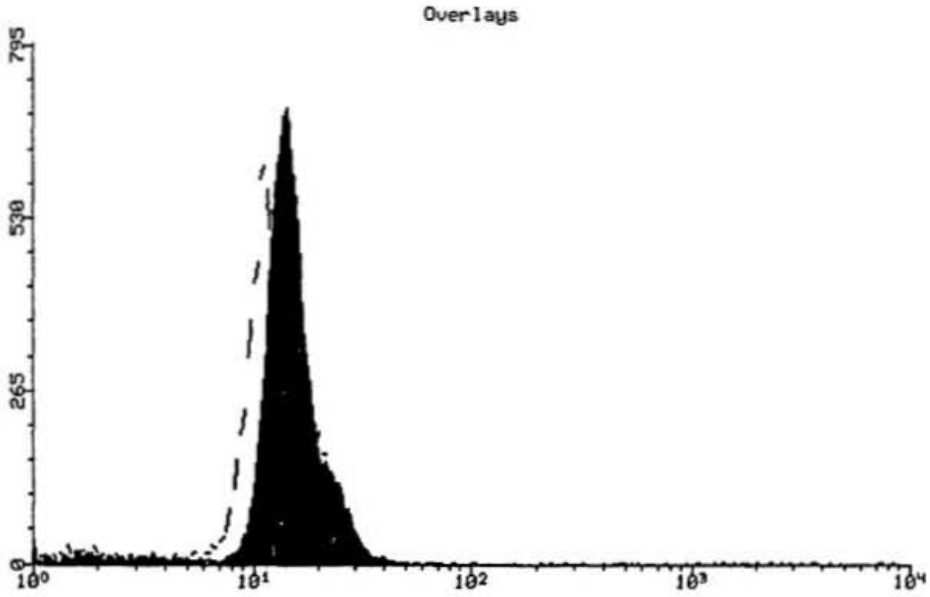
Although no positives were found in the non-bound population, only 33 wells were tested, making it difficult to draw any

conclusions as to the number of specific cells which escaped selection. As stated previously, an error in calculations was the cause of poor growth in this population.

3.2.2E FUSION NUMBER 15

This fusion was done using spleen cells from a mouse immunized with the transfectant DP8304 (DPβ1:0401). For this experiment, 65% of the fused population of cells was plated immediately following fusion (conventional population), with the other 35% grown as a bulk culture. On day 5 post-fusion the bulk culture was subjected to Ag-specific selection using DP-coated beads. Since NFLD.M58 does not react with DPβ1:0401, it was not useful for this experiment. Therefore only M67-coated beads were used for immune adsorbtion of DP molecules from lysates prepared using the BCL Jesthom (DPβ1:0401) and, because of this, hybrids expressing Ig receptors with the same specificity as M67 would escape selection. Following adsorbtion, the coated beads were stained with FITC-I3, and the results are shown in Figure 10. From these results it can be seen that the M67-DP-coated beads show a shift in fluorescent intensity relative to the negative control, indicating adsorbtion of DP molecules from the Jesthom cell lysate. Ten M67-DP-coated beads per hybridoma cell were then used for the selection experiment.

Rgn



Clear histogram: uncoated beads.
Solid histgram: DP-coated beads.

Figure 10. Flow cytometry results for detection of DP molecules, adsorbed from a Jesthom BCL lysate, on immunometallic beads coated with NFLD.M67 anti-DP mAb.

Following selection, the magnet-bound and non-bound populations were counted:

magnet-bound population: 1 x 10^4 hybridomas.

non-bound population: 4.5 x 10^5 hybridomas.

Therefore, 2% of the total number of hybrids were selected by the DP-coated beads.

The total population of magnet-bound cells, plus a portion of the non-bound population, were plated in 96-well plates, as before.

The results of the CELISA assay, which tested for Ab specific for the Jesthom cell line, are presented below:

	Conventional population	Magnet-bound population	Non-bound population
Total # wells with growth	1056	588	168
# and (%) +ve agst. Jesthom	16 (1.5%)	19 (3%)	1 (<1%)

These results show that the yield of Ag-specific hybridomas in the selected population was two times that of the conventional population. Non-specific binding was higher here than in previous experiments, with only 3% of the selected population being Ag-specific. For the non-bound population, 3 positives would be expected from 168 growing wells, based on the conventional population results. Only one positive was found, indicating that Ag-specific cells were less frequent, but not completely depleted, from this population.

This was the final immunomagnetic experiment done in this study. A discussion of the results will be presented in the following chapter.

3.3 SELECTION OF POST-FUSION HYBRIDOMAS USING PANNING

For this study, panning seemed to present an ideal method for selecting Ag-specific hybridomas. Since the immunizing transfectant cells spontaneously adhere to plastics, they provide a readily available source of solid-phase Ag. It was envisioned that the bulk culture of fused cells could be incubated on a layer of transfectant cells, during which time DP-specific hybridomas would bind, via their Ig receptors, to the DP molecules expressed on the surfaces of the transfectants. Subsequent washing would remove all cells that were not bound, leaving only Ag-specific hybridomas. Figure 11 gives a schematic diagram showing the principle behind this panning procedure.

A method was devised to test this procedure, as described in Materials and Methods section 2.3.1. 96-well plates were used for panning, so that the bound hybridomas could simply be left to grow on the layer of transfectant cells, eliminating the need to detach, count, and then plate the bound cells. For this reason it was necessary to irradiate the layer of transfectant cells before performing the panning procedure, in

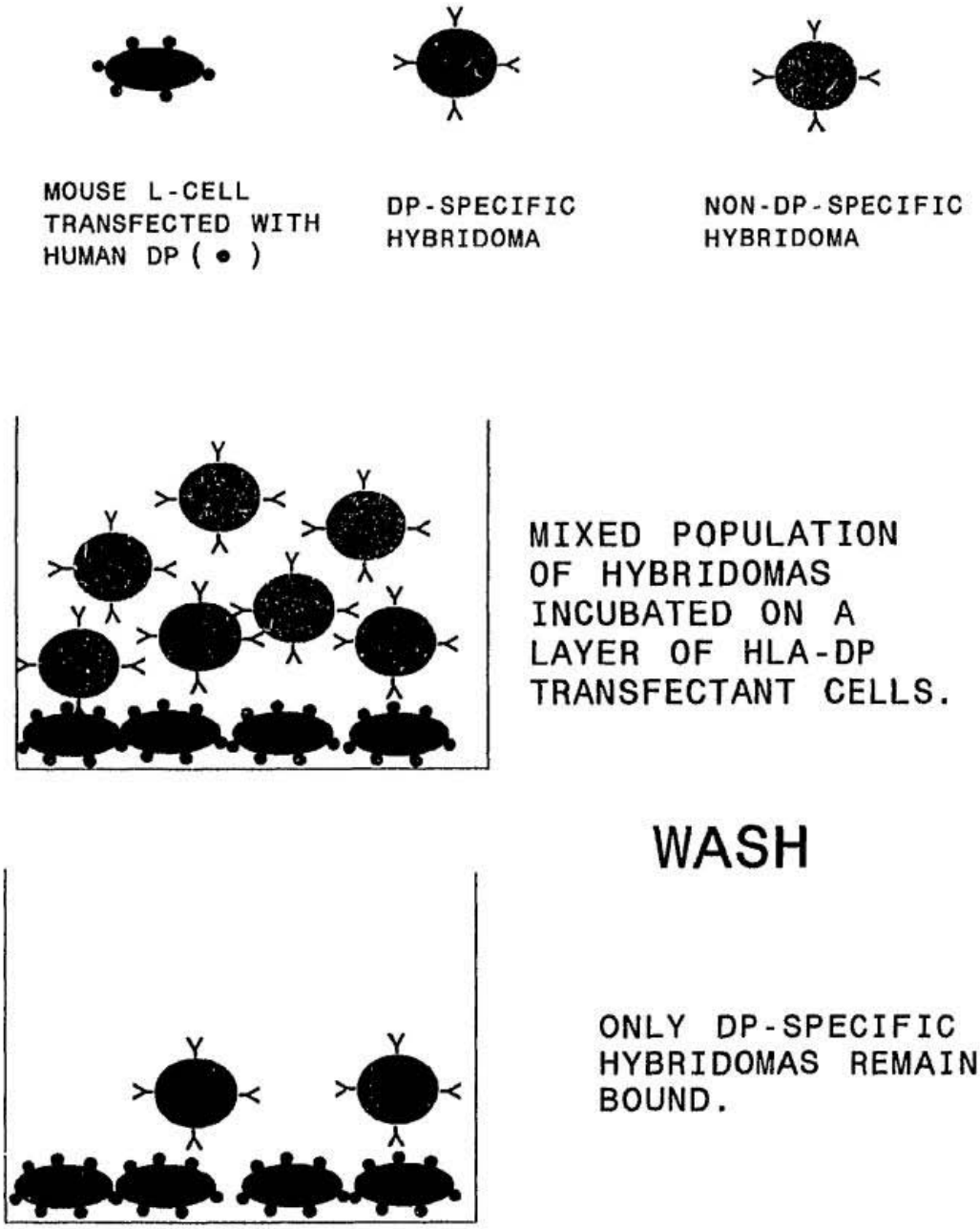


Figure 11. Schematic diagram illustrating the principle behind the panning selection procedure used in this study.

order to ensure that the transfectants would not multiply and overgrow the bound hybridomas. The incubation conditions that had been found to be optimal for selection using immunomagnetism (ie, 37°C for 30 minutes) were also utilized here.

3.3.1 PRELIMINARY EXPERIMENTS

Before beginning this panning procedure on bulk cultures of fused cells, there were several questions that needed to be answered:

1) Does irradiation of the transfectant cells affect their DP expression? If irradiation caused the transfectants to express lower levels of DP, then they would not be useful for selecting Ag-specific hybrids.

2) Will the transfectants adsorb significant amounts of any specific Ab being produced by the hybridoma cells? Since specific antibody will be reactive with the immunizing transfectant cell, these cells might adsorb the Ab present in the well, causing false negative results in the screening CELISA.

3) What is the best method for washing away unbound cells? The normal flick/washing procedure used in the CELISA assay could prove to be too harsh, causing bound cells to become detached. A more gentle washing procedure, in which the wash medium is

added and removed using a multipipettor, was considered as an alternative. However, this could result in a too many cells remaining non-specifically bound, and therefore a comparison of the methods was required.

3.3.1A CHECKING DP EXPRESSION ON IRRADIATED CELLS

For this experiment, the transfectant DP8306 was coated onto two 96-well plates, as described in Materials and Methods. The next morning, one of the plates was irradiated, using 1200 rads. Both plates were then run in a CELISA assay, with 10% DMEM added to four wells of each plate (negatives) and NFLD.M67 mAb added to another four wells (positives). The optical density results were averaged, and the negative control result was subtracted from the positive test result. This gave an optical density of 1.96 for the non-irradiated transfectants and 2.15 for the irradiated ones. Therefore, it was concluded that irradiation does not reduce the DP expression of transfectant cells.

3.3.1B CHECKING FOR ADSORBTION OF SPECIFIC Ab BY TRANSFECTANTS

For this experiment, one-half of a 96-well plate was coated with DP8306 transfectant cells. The next morning, this plate was irradiated, as before. Following irradiation, the plate

was washed, and NFLD.M67 mAb was added, in doubling dilutions, to four rows of the plate: two (duplicates) with transfectant cells and two (duplicates) without. A total of 100 μl was added to each well. This plate was incubated for 48 hours, and supernatant fluid from each well was removed and tested in a CELISA assay, against DP8306. The optical density (OD) results are presented in Table 11.

From these results it was clear that the transfectant cells would indeed adsorb some of the specific Ab present in the wells. To overcome this problem it was decided that all panned plates would be replicate plated, once the bound hybridomas had begun to multiply, in order to separate them from the layer of transfectant cells. Therefore, in all these experiments, replicate plating, using a multipipettor to transfer the content of the wells, was done on day 4-7 following the panning procedure. The original plates were then discarded.

3.3.1C TESTING THE WASHING CONDITIONS

In order to make this procedure feasible, it was decided that between 500 and 1000 hybridomas from the bulk culture population would be added to each transfectant-coated well, for panning. It was estimated, from previous DP fusions in our laboratory, that this number should give approximately one Ag-

Table 11: OPTICAL DENSITY (OD) RESULTS OF CELISA ASSAY TESTING ADSORBTION OF SPECIFIC ANTIBODY INCUBATED ON TRANSFECTANT-COATED VERSUS NON-COATED WELLS

Dilution of NFLD.M67 added	OD: transfectant-coated wells	OD: non-coated wells
undiluted	1.65	1.76
1:2	0.97	1.80
1:4	0.49	1.66
1:8	0.23	1.22
1:16	0.14	0.86
1:32	0.06	0.54
1:64	0.05	0.31

specific hybridoma per well. It was desirable to use the highest possible number of hybridomas per well, in order to minimize the number of plates, and thus the amount of labor, required for this procedure.

To test the washing conditions in relation to non-specific binding, 1000 SP2/O cells were added to each well of two DP8306-coated 96-well plates. Following incubation, one plate was washed by flick/washing x5, while the other plate was washed x3 using a multipipettor. The non-bound population was then collected and counted, and this count was used to indirectly determine the number of cells that remained non-specifically bound.

For the plate washed "gently", using a micropipettor, only 50% of the starting number of SP2/O cells were recovered in the non-bound population. This was taken to mean that approximately one-half the total number of cells added were sticking non-specifically to the wells. These conditions were obviously unacceptable.

For the plate washed by flick/washing, there were transfectant cells evident in the non-bound population, having become detached by the washing procedure, making an accurate SP2/O count difficult to obtain. Unbound transfectant cells become round, closely resembling SP2/O cells by light microscopy. All cells were counted, giving a number approximately 2x the starting number of SP2/O cells. This was taken to mean that

most of the SP2/O cells had washed off, plus a relatively small number of transfectant cells (approximately 2% of the number added to the wells).

Based on this experiment, it was decided to wash the panned plates x4 by the flick/washing procedure. Since an accurate non-bound population count could not be obtained using light microscopy, it was decided to estimate that a total of 10% of the starting population had remained bound to the wells, and, based on this number, to plate the non-bound population at 10 cells per well.

3.3.2 PANNING EXPERIMENTS - FIRST FIVE ATTEMPTS

The first five fusions utilizing panning to select Ag-specific hybridomas were done as described in Materials and Methods section 2.3.1. The details of these fusions can also be found in the Materials and Methods chapter. Between 500 and 1000 hybridomas were added to each transfectant-coated well, for each of these panning experiments. As for the immunomagnetism experiments, each fusion protocol resulted in three populations; a conventional population, a panned population, and a non-bound population (which was plated as described above). All growing wells from the three populations were tested for specific antibody production in a CELISA assay using the immunizing transfectant cell as the target. The

results are presented in Table 12, where column A gives the total number of wells with hybrid growth in each population, and column B gives the number of these wells that were positive against the immunizing cell in the primary CELISA assay, as well as (in brackets) the percent of total hybrids that this number represents.

It can be seen that, in each of the five experiments, an increased yield of Ag-specific cells was found in the panned population. The total numbers, reported in the last row of the table, show that Ag-specific cells were found, on average, twice as frequently in the panned populations, compared with the conventional populations. Looking at individual results, three of these five experiments gave frequencies six times, seven times, and eight times higher in the panned populations.

These results indicated at least some degree of sucess. However, several problems had become evident by this time. First, the non-bound populations were, in most cases, showing a fairly high number of Ag-specific hybridomas, indicating that a significant proportion of the specific cells were escaping selection. In addition, the non-bound populations were generally showing very poor, and much slower, growth than the bound populations. This indicated that a population considerably larger than the estimated 10% was remaining bound non-specifically to the panning plates. As this would certainly invalidate the results, it was to decided to

Table 12: RESULTS OF PRIMARY SCREENING CELISA FOR EXPERIMENTS UTILIZING PANNING TO SELECT POST-FUSION HYBRIDOMAS

Fusion Number	Conventional Population		Panned Population		Non-bound Population	
	A	B	A	B	A	B
6	624	60 (10)	85	54 (63)	93	17 (18)
7	192	12 (6)	83	41 (49)	15	2 (13)
8	1680	174 (10)	120	27 (23)	ND	ND
9	3248	1300 (40)	96	70 (73)	284	87 (31)
10	1816	125 (7)	93	45 (48)	15	0 (0)
Totals	7560	1671 (22)	477	237 (50)	407	106 (26)

Column A: Total number of wells with growing hybridomas.

Column B: Number and (percent) of wells positive against the immunizing cell in the primary screening CELISA.

ND : Not done.

Number of hybridomas added per well: 500 - 1000.

investigate this potential problem before proceeding.

3.3.3 ASSESSING THE DEGREE OF NON-SPECIFIC BINDING TO TRANSFECTANT-COATED WELLS

An experiment was performed to determine the number of SP2/O cells that remain bound to transfectant-coated wells following the panning technique utilized in the above experiments. SP2/O cells were plated in 96-well plates, using doubling dilutions, from 500 cells per well down to 4 cells per well. Twenty-four wells were plated on each dilution. Four identical sets of plates were included: the first set (A) used transfectant-coated 96-well plates and were not subjected to the washing procedure - they were simply left at 37°C following plating of the SP2/O cells, as a control for cell growth at the varying dilutions; the second set (B) used identical transfectant-coated 96-well plates, the full procedure was done, and the non-bound population was discarded. The third (C) and fourth (D) sets were identical to sets A and B, respectively, except that transfectants were not coated onto the 96-well plates. This was done to determine if non-specific binding was attributable to the transfectant cells or to the plastic.
All plates were maintained for 48 hours, at which time they were fed, and the transfectant-coated plates (A and B) were replicate-plated. After incubation for another three days, all

wells were examined microscopically to determine the number with SP2/O growth. The results are presented in Table 13. From these results it can be seen that non-specific binding occurred at approximately the same rate for both transfectant-coated and non-coated wells. Therefore it was the plastic, not the transfectants, to which the cells were binding. Sets A and C, which were not flick/washed, had 100% of wells with growth at all dilutions tested, showing that cell growth was not a problem. Sets B and D, which were subjected to the full washing procedure, had 80-100% of wells with growth at 500, 250, and 125 cells per well. Plating 62 cells per well resulted in approximately 50% of wells showing cell growth, while 31 cells per well gave 20% growth. Lower numbers of cells per well (16, 8, and 4) resulted in approximately 10% of wells with cell growth.

Therefore it was obvious that the experiments done so far, using 500-1000 cells per well, were likely to have had a high level of non-specific binding, making the results invalid.

3.3.4 PANNING EXPERIMENTS - LAST THREE ATTEMPTS

It was decided to continue with the panning experiments using only 50 hybridomas per well, as this number seemed to give an acceptable level of non-specific sticking. This low number of cells per well would increase the labor involved

TABLE 13: RESULTS OF EXPERIMENT TO ASSESS NON-SPECIFIC BINDING OF CELLS TO 96-WELL PLATES FOLLOWING THE PANNING PROCEDURE

# SP2/O cells added per well	# wells with growing SP2/O cells (maximum = 24 wells)			
	A	B	C	D
500	24	23	24	24
250	24	22	24	24
125	24	20	24	20
62	24	11	24	17
31	24	5	24	5
16	24	1	24	3
8	24	3	24	2
4	24	1	24	1

A: Transfectant-coated wells - control samples (ie, no washing).

B: Transfectant-coated wells - test samples.

C: Uncoated wells - control samples (ie, no washing).

D: Uncoated wells - test samples.

significantly, due to the large number of plates that would be required to accomodate the total bulk population of hybridoma cells. However, if this modified procedure worked - allowing isolation of specific cells with only a low level of non-specific binding - then an attempt could be made to improve the washing conditions, so that a higher number of cells per well could be added.

The results for the last three panning experiments are presented in Table 14, with the last row giving the total numbers. From these results it was quite obvious that DP-specific cells were not being isolated by this procedure; the total non-bound population gave a higher proportion of Ag-specific cells than the total panned population. At this point we decided to stop the panning experiments, as results indicated that the procedure was not suitable for selecting Ag-specific cells from post-fusion bulk cultures.

TABLE 14: RESULTS OF PRIMARY SCREENING CELISA FOR EXPERIMENTS UTILIZING PANNING TO SELECT POST-FUSION HYBRIDOMAS

Fusion Number	Conventional Population		Panned Population		Non-bound Population	
	A	B	A	B	A	B
11	720	78 (11)	180	7 (4)	130	12 (9)
12	880	266 (30)	190	11 (6)	200	73 (37)
13	2160	70 (3)	79	11 (14)	211	7 (3)
Totals	3760	414 (11)	449	29 (6)	541	92 (17)

Column A: Total number of wells with growing hybridomas.

Column B: Number and (percent) positive against the immunizing cell in the primary screening CELISA.

Number of hybridomas added per well: 50.

4.0 DISCUSSION AND CONCLUSIONS

This thesis represents an attempt to improve the efficiency of mAb technology, by selecting only the Ag-specific hybridomas from the total population resulting from cell-mediated PEG fusion. Fusion of immune mouse splenocytes with myeloma cells results in a vast majority of non-specific hybridomas. When the Ag is of low immunogenicity, such as the one used in this study (HLA-DP), Ag-specific hybrids may be very rare indeed. Conventional technology relies on limiting dilution microculture of the total post-fusion population to isolate the very few Ag-specific clones. Clearly, selection of these hybridomas prior to limiting dilution microculture would be a highly desirable improvement, resulting in increased efficiency of mAb generation.

An Ag-specific selection method, designed for experiments such as the ones described in this study, must meet two important criteria in order to be considered feasible:

1) The selection procedure must be fairly simple to perform. A labor- or time-intensive selection method could, in the long run, be more troublesome than conventional methodology, thereby defeating its purpose.

2) The selection procedure must be capable of isolating most of the Ag-specific hybridomas present. Since the generation of Ag-specific hybridomas in these experimental systems may be

very rare events, the selection method must be capable of isolating this small number of Ag-specific cells from a much larger population.

Two selection methods were attempted in this study - immunomagnetism and panning. The results obtained with each method will be discussed, particularly in relation to the two criteria above. First however, some important points, applicable to both selection methods, need to be addressed.

4.1 CELL NUMBERS

In this study, a total of 15 fusions were performed, using splenocytes from mice immunized with mouse transfectant cells expressing human DP. For these fusions the majority of the post-fusion population was plated in the conventional manner, with the remainder grown in bulk culture for subsequent selection attempts. The yield of Ag-specific hybridomas from the conventional, selected, and non-selected populations was then determined, to see if the selection procedure had resulted in an increased yield. Several points about the cell numbers involved here warrants clarification.

First, as a point of interest, it should be remembered that the Ag-specific yields reported in these experiments refer to the percentage of wells with growing hybridomas that contain Ab reactive against the immunizing transfectant cell. The vast

majority of these Ab, upon subsequent testing (not reported in this thesis), were found to react with all transfectant cells tested, including DR-expressing transfectants. The percentage showing specificity for DP, particularly for polymorphic determinants, was many-fold lower than the yields reported here. Although this point is not of importance for the results presented in this study, it does explain why a relatively high number of Ag-specific hybridomas were found in these experiments, when the antigen of interest was discussed previously as being of low immunogenicity.

Second, it is important to note that the yields of Ag-specific hybridomas reported for the three populations in each experiment done in this study are not directly comparable. In these experiments, yields are based on the number of wells, rather than the number of hybridomas, testing positive for specific Ab. Therefore, in order for a direct comparison to be made, the number of hybridomas seeded per well in each population would have to be the same. Although equal numbers per well was the aim of these experiments, results indicate that it was not always achieved.

For the conventional populations, the actual hybrid numbers per cultured well was unknown, as it is very difficult to determine the number of hybridomas formed immediately following fusion. In this study, the number plated per well was based on the spleen cell count, determined prior to PEG-

mediated fusion; 1.0 - 1.5 x 10^5 spleen cells were plated in each well following the fusion procedure. This concentration was already well characterized in our laboratory, to give hybridoma growth in close to 100% of wells for most fusion attempts, as it did for the ones reported in this study. Therefore, in the majority of these wells, growth must be oligoclonal, as opposed to monoclonal.

For the selection experiments, the bulk population was counted prior to the start of each experiment, to ascertain the number of hybridomas available for selection. It was hoped that the selected and non-selected populations could also be counted following the selection experiment, and that this count would then enable plating of the cells in such a way to give "approximately clonal" growth, similar to the conventional populations. For the immunomagnetic experiments this is, indeed, the procedure that was followed. The selected and non-selected populations were counted and plated at approximately three to six hybridomas per well. However, growth was often much poorer in these populations than in the corresponding conventional population, indicating that different numbers of cells had been seeded in the two populations. Inaccurate counting of the hybridomas is a possible explanation for this; counts were often difficult to perform, with so few hybridomas among large numbers of other cells.

For the panning experiments, as for the immunomagnetic

experiments, a count was obtained for the bulk population before the experiment was initiated. However, the number of hybridomas that had been selected by panning was not directly determined, as the bound cells were not eluted. It was thought that the non-selected population could be counted and, by subtracting this number from the total count, a good estimate of the number of hybridomas isolated by panning could be determined. However, accurate counting of the non-selected population was unattainable by simple light microscopy, due to the fact that transfectant cells were present in this population and could not be distinguished from hybridomas. Therefore, it was estimated that approximately 90% of the hybridomas were recovered in the non-selected population, which was then plated in an "approximately clonal" manner, as above. As for the immunomagnetic experiments, less than ideal growth often resulted for these panned populations.

It should be clear from this discussion that the actual number of hybridomas per well in each population is not known to be the same, making direct comparisons impossible. In addition, the frequency of Ag-specific hybridomas may be different for the conventional population (immediately following fusion) than for the bulk population (four to seven days post-fusion). Although it seems likely that all hybridomas in the bulk population multiplied at approximately the same rate, leaving the frequency of Ag-specific hybrids virtually unchanged over

the period of bulk growth, there is no evidence from this study to prove that this is true. It is possible that, having encountered Ag, the Ag-specific hybrids grow at a faster rate than the non-specific ones, giving increasing frequencies over the period of bulk growth. Alternatively, the Ag-specific cells may multiply at a slower rate, or may be selectively killed, giving a lower frequency in the bulk population. If any of these possibilities proved true, then comparisons between the conventional populations and the selection populations would be invalid.

Therefore, for these experiments, direct comparisons between the populations cannot be made. However, the selected populations should show a higher Ag-specific yield than the corresponding conventional populations, and, more importantly, the non-selected populations should show a very low (and preferably absent) frequency of Ag-specific cells.

4.2 SURFACE Ig EXPRESSION OF HYBRIDOMAS

The second point that needs to be addressed, and is applicable to both selection techniques attempted in this study, is the question of surface Ig expression by hybridomas. Do all Ab-secreting hybridomas express surface Ig? Does the level of expression vary and, if so, under what conditions? These are very important questions for this study, as selection, in both

techniques, is directly dependent on the expression of surface Ig by the Ag-specific cells. If some Ab-secreting hybridomas do not express sIg, then it would be impossible to select them from a post-fusion population using either of the two techniques reported here. If the expression levels vary, then it is important to know this, and to attempt selection when expression is at its highest.

4.2.1 IS SURFACE Ig EXPRESSED BY ALL Ab-SECRETING HYBRIDOMAS?

The question of whether all Ab-secreting hybridomas express surface Ig is a difficult question to answer. Studies indicate that the majority of B-cell hybridomas are positive for membrane Ig of the same specificity as the secreted mAb (Legrain et al, 1983; Martel et al, 1988). In addition, several studies have utilized cell surface Ig to isolate hybridomas with useful phenotypes; isotype switch variants (Baumhackel et al, 1982; Fauget and Agee, 1993) and higher avidity variants (Martel et al, 1988), in addition to Ag-specific hybridomas, have been identified and selected based on characterization of cell surface Ig. Studies on established hybridomas show a good correlation between antibody detected in supernatants and cell positivity for sIg (Gardner et al, 1985). Therefore it appears that most Ab-producing hybridomas do indeed express sIg. However, at least one report (Neuberger

and Rajewsky, 1981) describes a hybridoma that secretes an IgM Ab, but has no detectable sIgM. Hence, such hybrids do exist, although they may be rare.

It is tempting to speculate that hybridomas aquire the Ig phenotype of the B cell that fuses with the myeloma cell. Thus, hybrids which secrete antibody but do not express sIg would result from fusion of terminally differentiated plasma cells. Fusion of resting B cells would result in hybridomas which express, but do not secrete Ig, while the formation of expressing and secreting hybrids would require fusion of enlarged B lymphocytes which express sIg and actively secrete antibody of the same specificity. In human adults it has been determined that approximately 1% of circulating B cells are actively secreting Ig (Hougs et al, 1993). These cells were shown to be, not fully differentiated plasma cells, but enlarged B lymphocytes with a cytoplasm rich in rough endoplasmic reticulum. The majority of these cells expressed B lymphocyte markers and sIg, unlike fully differentiated plasma cells. It was also shown that the numbers of these cells could be increased considerably by systemic immunization. It is therefore conceivable that fusion of these cells results in hybridomas which express and secrete Ig. This, however, is pure speculation, and it is realized that fusion of two individual cells may result in formation of a hybrid which expresses phenotypes different from either of the

two cells from which it was derived.

Regardless of the reason, it is very possible that a minority of the hybridomas formed in any fusion will be secreting, but not expressing Ig. Selection procedures such as the ones used in this study, which depend on surface Ig expression, would therefore be incapable of isolating these non-expressing hybridomas. An interesting extension of the present study would be to examine positive wells from the non-selected populations to see if absent, or very low, expression of sIg could explain why these Ab-secreting hybridomas had escaped selection.

4.2.2 DOES THE LEVEL OF SURFACE Ig EXPRESSED BY HYBRIDOMAS VARY?

For the experiments performed in this study, knowledge of variations in the level of sIg expression by hybridomas was considered important; it would obviously be advantageous to attempt selection when levels are at their highest. Studies show that hybridoma sIg levels can vary significantly, for different reasons. First of all, Westerwoudt et al (1984) showed that at least 40% of Ab positive hybridoma clones lose their Ab-producing capacity during the first three weeks following fusion. This is due to chromosomal loss, which appears to occur at a high level immediately following fusion,

with more gradual segregation during continued culture. Therefore, in the experiments presented here, a significant number of selected hybridomas may subsequently lose their antibody producing capability, giving negative results.

Gardner et al (1985) studied frequencies of Ab-negative variants in clonal populations from established hybridomas. They found that cells not secreting Ab arise at rates as high as 0.06 per generation, and that this rate can vary by two orders of magnitude for independent clones. High frequencies of Ab-negative variants were found to indicate imminent loss of Ab-producing capacity. Parks et al (1979), also studying established hybridomas, found large differences in the Ag-binding capability among Ab-producing cells of a clone; even fresh clones contained a significant fraction (approximately 2.6%) of cells that did not express sIg, and did not secrete active Ab. In addition, it was indicated that some of these non-expressing cells could give rise to Ab-producing subclones.

Meilhoc et al (1989) studied the kinetics of Ab synthesis and secretion from a murine hybridoma cell line using measurements of total cell-associated Ig, surface Ig, and secreted Ig. Measurements were made at various times over a four day period. At all times, staining for sIg showed two peaks, indicating a population of cells with very low surface expression and a second population with high expression. The

staining intensity of the high population varied over time, and there were also indications that cells could move between the two populations. This study found that the rate of secretion does not correlate well with sIg; the secretion rate remained approximately constant during batch culture, while the sIg levels varied considerably. The sIg levels, however, were found to correlate with the total amount of cell-associated Ig. Based on their results, these investigators suggest that, during exponential growth of hybridoma cells, more Ig is synthesized than secreted, with the excess being targetted to the cell membrane. As the cells leave the exponential phase of growth, the rate of synthesis drops slightly below the rate of secretion; surface expression of Ig then decreases, to allow the rate of secretion to remain constant. If this is true of all hybridomas, then it may be important to ensure that these cells are in log phase at the time of selection. For the experiments in this thesis, exponential growth was encouraged by frequent feeding of the bulk culture.

Limited studies done on established hybridomas in our laboratory (unpublished) also show that there is considerable heterogeneity in sIg expression by Ab-producing cells of a hybridoma clone. All clones tested were found to express sIg, with a total loss of surface expression (seen in one clone) correlating with loss of antibody production. The sIg levels,

however, were found to vary greatly. Using direct staining with phycoerythrin-labelled GAM Ig and flow cytometry, only two of the ten clones tested showed a homogenous population of sIg expressing cells. Four others showed a heterogenous population, with expression levels ranging from very low to very high; the remaining four showed a bimodal staining pattern. Therefore, it seems that the sIg levels of hybrid cells can vary greatly. Cells producing significant quantities of antibody, but displaying low levels of sIg, are likely to be present in a clonal population at any given time. This indicates a need for high sensitivity in selection procedures that depend on sIg expression.

Although the studies discussed above give valuable information pertaining to the levels of sIg expressed by established hybridomas, no studies were found that examined sIg levels of newly-formed hybrids. It is quite possible that expression of surface immunoglobulin by newly-formed hybridomas is different from that shown by establihed clones. For example, there may be a lag period before Ig is expressed on the surface of a new hybridoma. It is also possible that there is a "burst" of sIg expression at some point following establishment of the new cell; if so, this would be the ideal time to attempt Ag-specific selection. During the course of this thesis, an attempt was made to study the surface Ig expression of hybridomas immediately following the fusion

procedure. Unfortunately, these experiments did not yield the desired information, and for this reason they were not formally reported as part of this study. However, since some pertinant information was revealed through these experiments, a brief discussion is presented below.

4.2.3 EXPERIMENTS TO STUDY sIg EXPRESSION BY NEWLY-FORMED HYBRIDOMAS

Determination of sIg levels expressed by newly-formed hybridomas is not a simple task, due to the fact that the number of hybrids resulting from a fusion is very small. In order to study the sIg expression of this small number of hybrid cells, it is necessary to isolate them from the total post-fusion population, and this presents a challenge. It was suggested that staining for DNA content would be a feasible approach to isolating hybridoma cells from a post-fusion population; since hybridomas are formed by fusion of two or more cells, they will have a greater amount of DNA than either unfused spleen cells or unfused SP2/O cells. Staining the post-fusion population for DNA content should then allow hybridomas to be distinguishable from all other cells present. This procedure was first attempted on a cell mixture designed to resemble a post-fusion population, in that it contained mouse spleen cells, SP2/O cells, and cells from an established

hybridoma. However, it differed from a post-fusion population in that equal numbers of all three cell types were present. The procedure that was followed involved staining for sIg first, using FITC-labelled GAM Ig. Subsequently, the cells were fixed with 95% ethanol, followed by DNA staining using propidium iodide. Flow cytometric analysis then revealed that the three types of cells could easily be distinguished. The spleen cells, with the lowest DNA content, contained a population of cells negative for sIg, and a second population that stained strongly for sIg. The SP2/O cells were found to have approximately twice as much DNA as the spleen cells, and were negative for sIg. The hybridomas showed approximately three times the DNA content of spleen cells, and were positive for sIg, with a staining intensity somewhat lower than that of the positive spleen cell population.

Once it had been ascertained that this procedure could indeed distinguish hybrids in a mixed population, two post-fusion bulk cultures were subjected to the same procedure, with samples being analyzed immmediately following fusion, and at 24 hour intervals. A control population, consisting of the same cells, but without exposure to PEG (and therefore devoid of hybridomas), was also included. Analysis revealed these populations to be much more heterogenous than the cell mixture used in the first experiment. In the post-fusion bulk cultures, a population that was considered to be hybridoma

cells was not detected until day 5 post-fusion. At this time, and for several days thereafter, these cells showed a homogeneous population when stained for sIg, with an intensity higher than that of the negative spleen cells, but considerably lower than the staining intensity of the positive spleen cell population. Therefore, at 5 days post-fusion, the hybrid cells appeared to be expressing moderate levels of sIg, and these levels remained constant over the next four day period.

Presumably, in order to use this procedure to study sIg expression of hybridomas immediately following fusion, many more cells would need to be collected during analysis. To do this, multiple mouse spleens would need to be combined into one post-fusion population, to ensure adequate numbers of hybridoma cells for detection by flow cytometry. This was not attempted during the course of this thesis, but would be an interesting and worth-while experiment for future consideration.

4.3 IMMUNOMAGNETISM RESULTS

Overall, the immunomagnetism experiments performed in this study indicate that Ag-coated metallic beads, used for selection of specific cells from post-fusion populations, results in increased yields of specific hybridomas, compared

to conventional fusion methodology.

In preliminary experiments, with GAM IgG-coated beads, it was first shown that an IgG-expressing population could be separated from other cells that do not express surface IgG (figures 5(a) and 5(b)). Next, in fusion experiments using these same beads, isotyping results (table 10) showed that there was a substantial enrichment of IgG-positive cells in populations selected by GAM IgG-coated beads. However, these experiments also showed that selection was incomplete, with a small number of specific cells remaining in the non-selected populations. As discussed above, this may be due to variations in the levels of surface Ig expressed by the hybridomas.

In the final two experiments, DP-coated beads were prepared and used for selecting specific hybridomas. Again, the results indicated that these beads do, indeed, enrich for Ag-specific hybridomas, but they do not result in isolation of 100% of the specific cells present.

It must be noted that, in these final two immunomagnetic experiments, preparation of the DP-coated beads was not a simple procedure, due to lack of readily available purified DP. A BCL expressing the appropriate DP type was grown and lysed, with the lysate then being used as the DP source for coating beads. Therefore, in relation to the two criteria discussed at the beginning of this chapter, pertaining to the feasibility requirements of Ag-specific selection methods, the

first criterion (simplicity of experimental design) was not satisfied for this particular system (ie, the labor required for bead preparation was excessive, compared to conventional methodology). However, for systems where purified Ag is readily available, bead preparation would be less of a problem, and this first criterion might be satisfied.

The second criterion (ability to select the majority of Ag-specific cells present) appears to be satisfactorily met by the immunomagnetic selection procedure used in this study. Although all specific cells available were not isolated, it seems that the majority were, particularly with the DP-coated beads. It is highly probable that isolation of 100% of the desired cells is unattainable by this procedure, due to variability in sIg expression levels. In conclusion, therefore, this study shows that Ag-coated metallic beads can provide a rapid and reliable means of selecting Ag-specific hybridomas from post-fusion bulk cultures, particularly when the antigen is readily available in purified form.

4.4 PANNING RESULTS

Panning presented a seemingly ideal method for selecting DP-specific hybridomas from post-fusion populations, due to the ease with which it could be adapted to the experimental system. The immunizing transfectant cells, which are easily

grown to large numbers, express human DP molecules on their surfaces. These cells also adhere spontaneously to plastics and thus provide a readily available source of solid phase DP. At first, this procedure looked very promising. Results for the first five experiments (Table 12) indicated that panning was indeed resulting in a significantly higher yield of DP-specific hybridomas. However, by this time it was also becoming evident that a significant number of specific cells were escaping selection (as evidenced by the number of positives in the non-selected populations) and that non-specific hybridomas were adhering to the wells at a higher rate than expected (as evidenced by the slower growth seen in the non-selected populations). An experiment done to estimate the extent of non-specific cell binding (Table 13) indicated that, at the hybridoma concentration used in the first five panning experiments (500-1000 per well), non-specific adherence was occurring at a high level. This realization virtually invalidated the results for these five panning experiments; since hybrids were sticking to the plates non-specifically at a high rate, the selected populations would have been far from clonal, and therefore not at all comparable to the conventional and non-selected populations.

The results of this experiment indicated that, in order to achieve "approximately clonal" conditions, the number of hybridomas added to each transfectant-coated well needed to be

reduced to less than 100. This modification lessened the feasibility of the panning procedure, due to the large number of plates that would be required for panning a post-fusion population at this low concentration of hybridomas. However, it was decided to proceed with this modified experimental protocol because, if panning was indeed isolating the majority of the Ag-specific cells, then it was possible that the washing conditions could be modified to reduce non-specific adherence, and thus the number of hybridomas added to each well could then be increased.

Table 14 gives the results for three panning experiments that were done using 50-100 hybridomas per well. For these experiments, the non-selected populations showed a higher frequency of Ag-specific cells than the selected populations, showing that Ag-specific hybridomas were not remaining bound to the transfectant-coated wells. At this point the panning procedure was abandoned.

The reason for the failure of this procedure is not entirely clear. The literature search presented in Chapter 1 revealed three other studies in which panning was utilized for Ag-specific cell selection, for the purpose of mAb production. Although these studies used procedures which differed significantly from the one utilized in this study, the basic ideas were the same, and the general experimental designs were similar. All three experiments resulted in generation of Ab-

producing cell lines; however, the success of the panning selection itself appears limited. In the first study (Winger et al, 1983) only the population selected by panning was reported; no comparison with unmanipulated or non-selected populations was included. Therefore it is impossible to evaluate the success of the panning procedure used in this study. The other two studies were from the same investigators (Steenbakkers et al, 1993 and 1993a), and, based on their results, they estimated that only between five and ten percent of the total number of Ag-specific cells were isolated by the panning procedure used in their experiments. This is a very low percentage; the great majority of Ag-specific cells had escaped selection in these two studies, as was seen in the experiments reported here. The authors then went on to suggest that the panning procedure preferentially selects cells expressing high-affinity Ig receptors. It would be of interest to explore this theory in experiments such as the ones reported in this thesis. Binding studies on mAb from a random sample of positive wells from both the selected and the non-selected populations would reveal if higher-affinity cells had indeed been selected. If so, this procedure could prove useful for those cases where the Ag is not of low immunogenicity, and where the "best-fitting" mAb are desired.

In relation to the two criteria discussed earlier, pertaining to feasibility requirements for Ag-specific selection methods,

the panning procedure used in this study certainly meets the first requirement (simplicity of experimental design), but falls very short for the second requirement (abilty to select the majority of Ag-specific cells present).

In conclusion, therefore, this study shows that panning, using the procedure described here, is not a useful method for isolating Ag-specific hybridomas from post-fusion bulk cultures. It should be noted, however, that panning may be useful for isolating Ag-specific hybrids that express high-affinity Ig receptors.

4.5 FUTURE WORK

In my opinion, the most interesting extension of the work done in this thesis would be an investigation into the expression levels of surface immunoglobulin by antibody-secreting hybridomas. Since antigen-specific selection methods, such as the ones described in this thesis, intimately depend on hybridoma sIg, such knowledge is thought to be necessary to ensure successful selection of all specific cells available. Evidence was presented which showed that sIg expression by established hybridoma clones can vary greatly. Therefore, it may be possible to manipulate sIg levels by experimental means. It is conceivable that a particular additive to the growth medium of a hybrid would result in increased surface

expression, if only for a short period of time. Cytokines come to mind immediately as candidates, although it would be interesting to examine a wide variety of additives. Ideally, one would discover a substance which reversibly arrests the secretion of antibody (to alleviate worry of the selecting antigen becoming saturated with free antibody), directing all manufactured antibody to the cell surface. In this way, even hybrids which secrete but do not express Ig would become capable of selection by these methods.

A timed study of the sIg levels expressed by several Ab-secreting hybridomas, grown under different conditions, or with specific additives, might reveal a way to switch the sIg level of expression to "maximum" in all Ab-secreting cells, for a short period of time. Such experiments would not be difficult to perform, so it is conceivable that an extensive array of conditions and/or additives could be examined over a reasonable amount of time. If successful, such knowledge would be useful for many circumstances, whenever high levels of sIg are desired. For antigen-specific selection techniques, such as the ones described in this thesis, such a discovery would make their probability of success many-fold higher.

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