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Increasing the Efficiency of Monoclonal Antibody Production by Isolating Splenic B Cells in Advance of Hybridoma Fusion

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Increasing the Efficiency of Monoclonal Antibody Production by Isolating Splenic B Cells in Advance of Hybridoma Fusion

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ABSTRACT

The process of producing monoclonal antibodies using the hybridoma technology has been an arduous task for many years. In this study, we aim to improve the efficiency of monoclonal antibody production by isolating mature splenic B cells in advance of fusion using magnetic activated cell sorting (MACS) to select for cells expressing CD138, a cell marker present in mature B cells. Pre-isolating a population of cells expressing $CD138^{+}$, but lacking CD19 and B220, resulted in a reduced number of cells required for fusion.

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LIST OF ABBREVIATIONS

ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
APC	Allophycocyanin
Fc	Fragment Crystallizable Region
FITC	Fluorescein isothiocyanate
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
mAb	Monoclonal Antibodies
MACS	Magnetic-Activated Cell Sorting
PBS	Phosphate buffered saline
PE	R-Phycoerythrin
PEG	Polyethylene glycol
R-PE	R-Phycoerythrin
SN	Supernatant

INTRODUCTION

The overall objective of this study was to improve the efficiency of monoclonal antibody production by separating splenic B cells in advance of fusion. It was predicted that isolating mature B lymphocytes prior to the fusion would remove unwanted non-B cells, decrease the number of cells to be fused, and reduce the number of irrelevant hybridomas, thereby increasing efficiency and reducing laborious screening. The isolation of B lymphocytes was done using magnetic activated cell sorting (MACS) while selecting for $CD138^+$ cells, a marker for mature B cells.

The commercially available $CD138^+$ Plasma Isolation Kit used for this experiment allowed for the isolation of cells expressing CD138 while expressing low amounts of CD45R (B220) and CD19. CD 138 is a heparin sulfate-rich integral membrane proteoglycan found on the surface of epithelial cells in mature mouse tissues, which actively participates in cell binding and cell signaling. This marker also behaves as a matrix receptor for interstitial collagens, fibronectin, and thrombospondin. When B lymphocytes emigrate to the peripheral circulatory system, expression of CD138 is temporarily lost, then it is re-expressed when B lymphocytes differentiate into plasmablasts and plasma cells, the desired stage for hybridoma production. CD19 is a protein expressed on immature B cells. Upon maturation into plasma cells, the presence of CD19 is lost. CD 45R (B220) is an isoform of the CD45 protein present on B lymphocytes throughout their development. Similar to CD19, expression of B220 decreases as cells differentiate into plasma cells. Using the $CD138^+$ Plasma Isolation Kit, it was possible to isolate a population of mature B cells that also produce antibodies. Pre-

isolating a population of cells expressing $CD138^{+}$, but lacking CD19 and B220, resulted in a reduced number of cells required for fusion.

BACKGROUND

Monoclonal Antibody Production

Therapeutic monoclonal antibodies generated using hybridoma technology first entered clinical studies in the 1980s (Reichert and Rosenweig, 2005). As of 2005, more than 150 monoclonal antibody products had been introduced into clinical studies. In their 1975 journal article describing the development of the hybridoma technology, Kohler and Milstein discussed the difficulty in producing antigen-specific monoclonal antibodies. In Kohler and Milstein's first experiment, only 3% of the hybrid clones were found to be antigen-specific. In their second experiment, the percentage of antigen-specific clones reduced to only 0.2% (Kohler and Milstein, 1975). From these numbers, it was clear that obtaining a hybrid cell that produces antibodies against a specific antigen is very difficult and infrequent; approximately 1 in 10^6 cells (Margulies, 2005). Since 1975, researchers have tried to improve the efficiency of monoclonal antibody development using techniques to isolate antigen-specific B cells prior to the fusion. Such techniques were expected to reduce the number of irrelevant hybridomas produced from the fusion, thus reducing laborious screening (Napaporn et al., 2009). A number of these techniques involve using isolation methods based on cell surface Ig expression. These techniques include capturing B cells on an antigen-coated solid matrix, using flow cytometry with fluorescently labeled antigen, and rosetting with magnetic particles or antigen-coated red blood cells (Kodituwakku et al., 2003).

Isolating antigen-specific B cells is an arduous task, as the number of such cells is less than 1% of the total number of plasma cells (Alkan, 2004). The success of an isolation can be evaluated by measuring the cell viability, reproducibility, yield, and

purity of the isolated B cells (Kodituwakku et al., 2003). The yield can be defined as the percentage of antigen-specific cells isolated from the initial number of cells. Purity can be defined as the percentage of antigen-specific B cells from the population of B cells. A problem that occurs with determining purity is distinguishing between specific versus non-specific B cell binding to the antigen. In order to overcome this obstacle, scientists have looked for methods to confirm specific binding. To date, no technique has been developed which allows for the isolation of a pure antigen-specific B cell population without also including non-specific B cells (Kodituwakku et al., 2003).

Isolation of Antigen-Specific B Cells Using Columns and Plates

One of the first attempts of isolating antigen-specific B cells was performed in 1968 using an antigen-coated solid matrix (Wigzell and Anderson, 1968). Wigzell isolated B cells by passing cells from the lymph node of an immunized mouse through an antigen-coated plastic bead column (Wigzell and Anderson, 1968). Using this procedure, Wigzell obtained yields of 60-95%. Specificity of the isolated B cells was determined using the haemolytic plaque assay developed by Neils Jerne. The purity of the isolated cell population was not determined for this experiment. After removing the bound cells from the column, it was found that the cell viability had reduced. Further analysis of the isolated cell population showed that although the column had been successful in isolating antigen-specific B cells, large amounts of non antigen-specific cells were also included. In 1993, Steenbakkers et al. used a variation of this technique to develop hybridomas (Steenbakkers et al., 1993). Steenbakkers immunized mice with HIV antigens. Isolation of the B cells was done by panning antigen-coated culture plates. The isolated cell

population was then fused to myeloma cells and cultured. The yield and purity of this experiment were calculated to be 5% and 24%, respectively. Subsequently, De Wildt et al. isolated antigen-specific B cells from patients diagnosed with systemic lupus erythematosus (SLE) (De Wildt et al., 1997). De Wildt incubated U1 ribonucleoprotein (U1 RNP) auto antigen-specific B cells in U1 RNP-coated wells, and used trypsin to remove the bound cells. The cells were then harvested and studied. Unfortunately, analysis of the supernatant revealed that only 0.5 to 1.5% of the antibody secreted by the isolated cells was specific to U1 RNP. The low purity could result from the presence of non-specific binding of cells to the coated wells in the final isolated population. Thus, although use of an antigen-coated solid matrix can isolate antigen-specific B cells, experiments using this technique have not excluded non-specific binding cells.

Isolation of Antigen-Specific B Cells Using FACS

In the 1970's, a new technique using fluorescent labeled antigens to isolate antigen-specific B cells was developed by Julius *et al*. In their experiment, spleen cells from limpet haemocyanin (KLH) immunized mice were labeled using immunofluorescence, then separated using flow cytometry (Julius et al., 1972). This technique is also termed fluorescence activated cell sorting (FACS). Analysis of the isolated B cell fraction indicated that 40–52% of the fluorescent-conjugated KLH antigen-binding cells had been isolated. However, purity was not measured in this study, indicating the possibility of a large number of non-specific binding cells being included in the isolated population. Hoven *et al*. (1989) used this FACS technique to isolate ovalbumin (OA)-specific B cells from immunized mice. The yield of this isolation was

1% and the purity was 42% (Hoven et al., 1989). After performing an ELISPOT analysis on the supernatant, it was found that only 20-50% of the isolated antigen-specific B cells produced antibody. In another experiment, Mcheyzer-Williams *et al*. (2000) used the same technique to study memory B cell development and maintenance by isolating memory B cells from immunized mice that expressed B220 and CD138 (Mcheyzer-Williams et al., 2000). After isolation of $CD138^+$ B cells using flow cytometry, ELISPOT assays showed that only 55% of the isolated $CD138^+$ antigen-specific B cell population secreted antigen-specific antibodies. Though the yield was not calculated, McHeyzer-Williams concluded that if the results of the ELISPOT reflected the actual purity of the isolated B cell population, this percent of purity is low (Kodituwakku et al., 2003). Thus, like the previous column and coated well techniques, flow cytometry has the ability to isolate antigen-specific B cells from the initial cell population but still does not produce high yield or purity.

Isolation of Antigen-Specific B Cells Using Rosetting

The use of rosetting with antigen-coated red blood cells began in the 1970's (Kodituwakku et al., 2003). This technique involves incubating lymphocyte cells with antigen coated red blood cells to form rosettes. Rosetted cells are then separated from the remaining cell population by means of sedimentation. In an experiment performed by Brody, spleen cells and bone marrow cells from mice were incubated with red blood cells from sheep (Brody, 1970). Rosettes were isolated using sedimentation, and the specificity of the isolated B cells was confirmed using haemolytic plaque assays. Neither the yield nor the purity were determined, but it was concluded that rosetting with antigen-coated

red blood cells was not a very effective method of isolating cells due to the difficulty of separating rosetted cells from the non-rosetted cells even after sedimentation (Kodituwakku et al., 2003). In 1977, Walker *et al.* modified the rosetting technique, using negative selection to remove non-antigen-specific cells from the initial population (Walker et al., 1977). This modification resulted in rosettes being formed around the non antigen-specific B cells. Walker's technique proved to be advantageous as it allowed for antigen-specific cells to be isolated without having to bind to other cells to form rosettes.

Isolation of Antigen-Specific B Cells Using Rosetting and Magnetic Fields

Since the 1980's, antigen-coated magnetic particles have replaced the red blood cells in a new rosetting technique. This magnetic technique involves forming rosettes using immunomagnetic beads, then uses a strong magnetic field to separate the rosetted cells from the non-rosetted cells (Kodituwakku et al., 2003). Egeland et al. first used this technique in 1988 to isolate rheumatoid factor (RF)-positive B cells from the blood of patients diagnosed with rheumatoid arthritis and from normal patients (Egeland et al., 1988). Egeland used magnetic particles to form rosettes with the RF specific B cells. Despite the low number of RF-specific cells in the body, using the immunomagnetic beads produced a high yield of RF positive cells. In addition, analysis by ELISA indicated that more than 92% of the isolated cells produced RF antibodies. In 1995, Irsch *et al.* developed a new technique called magnetic activated cell sorting (MACS) which allowed for the isolation of rare antigen-specific B cells (Irsch et al., 1995). In this experiment, cells were labeled with antibodies along with magnetic microbeads and phycoerythrin. Magnetic columns were used to separate the antigen-specific binding cells

and results were analyzed using flow cytometry. Using this procedure resulted in a purity of 75%; yield was not calculated. A few years later, in 1999, Leyendeckers *et al.* isolated memory B cells from immunized humans using a two step process involving MACS followed by flow cytometry. By using a combination of the two techniques, Leyendeckers was able to obtain a yield of 98% and a purity of 10% (Leyendeckers et al., 1999).

Thus, while rosetting techniques give a decent yield, a combination of MACS and flow cytometry have shown to give higher yields and purity. Isolating antigen-specific B cells using just immunomagenetic methods had once been a satisfactory method; however, using magnetic cell sorting (MACS) followed by fluorescence activated cell sorting (FACS) has become an efficient and reliable method of isolating antigen-specific B cells (Kodituwakku et al., 2003). The procedure described by Leyendeckers *et al.* has proven to be an efficient way of obtaining both a higher yield and higher purity. However, even with a combination of these techniques, the highest reported purities are still not 100%, indicating that isolating pure antigen-specific B cells still remains a difficult task.

Isolating Antigen-Specific B Cells Prior to Fusion

Since the development of the hybridoma technology, attempts to improve the efficiency of monoclonal antibody production have been made by isolating antigen-specific B cells *prior* to fusion. The solid matrices (columns or wells) and flow cytometry techniques used for this purpose are similar to those discussed above for isolating antigen-specific B cells after fusion. Experiments performed using the three different

techniques have huge discrepancies when reporting purities. With respect to purity, initially, low purities were reported as scientists used plaque forming assays to determine the purity of the isolated B cell populations. This assay can be used for determining antigen-specific B cell purity by counting the plaques that are formed when the antibody producing cells lyse the red blood cells. While the plaque forming assay ensures specificity, it is an inefficient method of determining purity since it is possible that only a small proportion of the isolated antigen-specific B cell population may differentiate into antibody secreting cells (Kodituwakku et al., 2003). Researchers have reported purities ranging from 0.5% to 88% when using the cell capture on antigen-coated solid matrix method. Purities of 2.5% to 90%, and 0.1% to 100%, have been reported using flow cytometric cell sorting and rosetting techniques, respectively. Such varied ranges in purity may be due to the differing methods used to calculate the purity of the isolated population or the variability of antigens being tested.

Similarly, all three techniques have shown wide variations when reporting yields. Yields of 5% to 95% have been reported when using cell capture on an antigen coated solid matrix, and yields of 5% to 55%, and 0.1% to 98%, have been reported using flow cytometric cell sorting and rosetting techniques, respectively. In addition, the characteristics of the different types of B cells may also have been a cause for such large discrepancies. For example, studies show that circulating B cells are not able to differentiate into plasma cells as quickly as marginal zone B cells (Kodituwakku et al., 2003). Hence, the type of B cell being used for each experiment should be taken into account when comparing the yield and purity obtained using the different techniques. Studies show that pre-enriching a population of antigen-specific B cells *prior* to fusion of

the B cells with myeloma cells does not necessarily increase the efficiency of the process, but does reduce downstream screening. Despite the advancements in technology, isolating a population of pure antigen-specific B cells remains a difficult task and efforts are constantly being made to increase the yield and purity of the isolated B cell population.

PROJECT PURPOSE

Using the solid matrix or flow cytometry techniques mentioned above, it is possible to isolate a population of antigen-specific B cells expressing a specific marker of interest. Although a newer technique, magnetic activated cell sorting (MACS), has been used to isolate antigen-specific B cells *after* fusion, to our knowledge no study has tested whether isolating antigen-specific B cells by MACS *prior* to fusion increases the efficiency of the process or reduces downstream screening efforts. In this study, the MACS principle was used to isolate plasma cells expressing CD138, a marker for mature B lymphocytes. It was predicted that isolating mature B lymphocytes in general (without isolating antigen-specific cells) prior to the fusion would significantly decrease the number of cells being fused, thereby increasing the efficiency of monoclonal antibody production, and potentially decreasing the downstream screening time.

MATERIALS AND METHODOLOGY

1.1 Materials

Mice

BALB/cJ and A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Abbott Bioresearch Center (ABC) animal facility. Immunizations were done following IACUC protocol #21. For the first fusion, mice were immunized with human IgG as a general immunogen to test mouse antibody formation against a human protein. For the second fusion, mice were immunized with 293 cells transfected with RON Delta 160 antigen.

MACS

The MACS $CD138^+$ Plasma Cell Isolation Kit for mouse was ordered from Miltenyibiotec (Catolog no. 130-092-530).

Other Reagents

B220-Mouse CD 45R R-PE antibody (Caltag laboratories, CAT# RM 2604 Lot#1389418B); Mouse CD19 FITC antibody (Caltag laboratories, CAT# RM 7701 Lot#0600B); Rat anti mouse CD138 APC antibody (BD Pharmigen, CAT# 558626 Lot#93862); R-PE conjugated affinity purified goat anti mouse IgG (1+2a+2b+3) Fc specific (Jackson ImmunoResearch, CAT #115-115-164 Lot#84859); Goat anti mouse Fc HRP (Immunopure, CAT #31439 Lot#JJ1172524); RPMI 1640 (Invitrogen CAT# 11875-093); Fetal Clone 1 (Hyclone CAT# SH 30080-03 Lot# ANM 20274);Ultra low

IgG Fetal Bovine Serum (Invitrogen CAT#16250-078,Lot#1215199); Azaserine Hypoxanthine (SigmaCAT#A9666); TMB (3,3',5,5',Tetramethylbenzidine (Sigma CAT#T0440); 2N H_2SO_4 (VWR CAT# VW3500-1).

1.2 Methodology

1.2.1 Isolating $CD138^+$ cells using Magnetic Activated Cell Sorting (MACS)

Spleens from immunized animals were removed, and single cell suspensions were prepared and washed. Cells were counted using crystal violet and a hemocytometer. After resuspending 10^8 cells in 400 μls of FACS buffer (PBS, 0.5% BSA, and 2 mM EDTA), 100 μl of Non-Plasma Cell Depletion Cocktail was added. Cells were mixed and incubated at 4-8^{o}C for 10 minutes, washed by adding 0.5-1.0 ml of buffer, and centrifuged at 300xg for 10 minutes. Cells were re-suspended in 900 μl of FACS buffer and 100 μl of Anti-Biotin Microbeads were added, followed by incubation at 4^{o}C for 15 minutes. The cells were once again washed and resuspended in 500 μl of buffer. The suspension was placed in an LD Column in the magnetic field of the MACS separator. The unlabeled cell fraction that passed through the column was collected. The eluent contained the pre-enriched plasma cells. This fraction was labeled LD Elution. Remaining pre-enriched plasma cells were collected by adding 2 ml of buffer to the column and collecting the eluent. To obtain the non plasma cells for the FACS analysis, 2 ml of MACS buffer were pipetted into the column and the fraction was flushed out using a plunger. This fraction was labeled LD fraction. The fraction containing the pre-enriched plasma cells was centrifuged at 300xg for 10 minutes and resuspended in 400 μL of

buffer. 100 μL of CD138 Microbeads were added and the cell suspension was incubated at 4-8^{o}C for 15 minutes. Cells were washed and resuspended in 500 μl of MACS buffer. Cell suspension was applied onto an LS column and the unlabeled cells that passed through the column were collected. The column was washed by adding 1 ml of buffer and collecting the eluent. This fraction, labeled LS Elution, contained CD138^{-} cells. The column was then taken out of the magnetic field and placed on a suitable collection tube. 2 ml of MACS buffer were added to the column and the fraction was flushed out using a plunger. This fraction, labeled LS Fraction, was expected to contain the CD138^{+} cells.

1.2.2 *Analysis of MACS Separation using FACS*

The various fractions collected from the MACS separation were screened using FACS. 10^{6} spleen cells from each fraction were counted using trypan blue and a hemocytometer. Cells were then placed in eppendorf tubes containing 1 ml PBS with 1% rat serum. 10 μl of diluted staining antibody were added to each fraction. Antibodies were diluted 1:100 in PBS. Four positive controls were used: cells only, cells with CD19-FITC, cells with CD138-APC, and cells with B220-PE. Fractions and positive controls were incubated at 4^{o}C for thirty minutes and then read using the FACS machine.

1.2.3 *Cell Fusion*

Spleens from immunized animals were removed and single cell suspensions were prepared. SP2/0 myeloma cells were harvested from culture and washed. Cells from each fraction and tumor cells were mixed in a ratio of 5 spleen cells to 1 SP2/0 cells. Cells were fused using 50% PEG 1000 using standard techniques (Kohler and Milstein,

1975). Fused cells were seeded in 96 well plates with selective media at various densities of spleen cells per well. Fusions were incubated at 37^{o}C for 7-10 days. When macroscopic colonies were observed, supernatants were removed and tested using ELISA and FACS. The amounts of spleen cells, Sp2 cells, PEG, and SFM used for each fusion are shown in Tables 3 and 4.

1.2.4 Screening for Antigen Reactivity using ELISAs

The supernatants from Fusion-1 were screened for specific reactivity using an ELISA. ELISA plates were coated with 100 μl of 1.0 μg/ml human IgG, incubated overnight at 4^{o}C, washed 3x with 200 μl TPBS, and blocked in 5% non fat dry milk in TPBS at room temperature for 1 hour before the assay. Supernatants from fusion were diluted 1:3 serially in PBS containing 1% BSA. After an hour of incubation at room temperature, the plates were washed three times with TPBS. 50 μl of 1:10,000 diluted goat anti mouse Fc HRP secondary antibody were added to each well and incubated at room temperature for one hour. The plate was washed 3x in TPBS and 100 μl of TMB substrate per well were added for colometric development for 5 to 10 minutes at room temperature. The reaction was stopped by adding 100 μl of 2N H_2SO_4. The OD 450nm was obtained by plate reader and data was recorded in notebook.

1.2.5 *Screening for Antigen Reactivity using Fluorescence Activated Cell Sorting (FACS)*

The supernatants from Fusion-2 were screened for antigen reactivity using FACS analysis. BaF3 cells were placed on 96-well plates and washed with PBS. Samples

(positive control antibodies and the supernatant from the fusions) were diluted 1:2 in PBS and incubated at 4^{o}C for one hour. After primary antibody incubation, the cells were centrifuged, washed once with PBS, and incubated with 50 μl of 2.0 μg/ml PE-conjugated affinity purified goat anti-mouse Fc specific antibody at 4^{o}C for one hour. After secondary antibody incubation, the cells were washed once with PBS and suspended in 50 μl of PBS/well. The plates were stored in 4^{o}C until read using the FACS machine.

1.2.6 Determining Mouse IgG Production of Hybridomas using ELISA

The supernatants from Fusion-1 and -2 were screened for the presence of IgG using ELISAs. ELISA plates were coated with 100 μl of 1.0 μg/ml goat anti-mouse IgG overnight at 4^{o}C ,washed 3x with 200 μl TPBS, and blocked in 5% non fat dry milk in TPBS at room temperature for 1 hour before the assay. Supernatant from fusions were diluted 1:2 serially in PBS containing 1% BSA, beginning at 1:5 dilutions. After an hour of incubation at room temperature, the plates were washed 3x with TPBS. 50 μl of 1:10,000 diluted goat anti mouse Fc HRP secondary antibody were added to each well and incubated at room temperature for one hour. The plate was washed 3x in TPBS and 100 μl of TMB substrate per well were added for colormetric development for 5 to 10 minutes at room temperature. The reaction was stopped by adding 100μl of 2N H_2SO_4. The OD 450nm was obtained by a plate reader.

RESULTS

The objective of this study was to improve the efficiency of monoclonal antibody production, or reduce downstream screening efforts, by isolating a subpopulation of antibody-producing plasma cells in advance of fusion. CD 138 is a marker found on the surface of epithelial cells in mature tissues. CD138 is present when B cells differentiate from plasmablasts into plasma cells, thus CD 138 represents a marker for mature B cells (presumably those secreting antibody). Using positive selection by magnetic activated cell sorting (MACS), $CD138^+$ cells were isolated from the spleen, then fused with SP2 myeloma cells. Fused cells, hybridomas, were cultured for two weeks and monitored for macroscopic colony growth, general IgG production, and reactivity to a specific antigen (when mice were pre-immunized).

The success of the MACS separation was determined using cell counts and FACS analysis. Tables 1 and 2 show cell counts from two separations done using MACS. In the first analysis (**Table 1**), 2.0 $x10^8$ splenic cells were obtained from two spleens. After labeling the cells with Non-Plasma Cell Depletion Cocktail (to remove non-plasma cells), Anti-Biotin microbeads, and passing the suspension through an LD column, 8.6 $x10^6$ pre-enriched general plasma cells were obtained. This represents 4.3% of the original number of cells. Labeling the pre-enriched plasma cells with CD138 microbeads and passing the suspension through a LS column, resulted in 0.5 $x10^6$ cells being obtained. This number is 0.25% of the original population of cells. Similar to the first analysis, the final fraction of the second analysis (**Table 2**) contained 0.27% of the starting number of cells. Both these analyses indicate that the majority of non-plasma cells and undifferentiated B cells were removed through the MACS separation.

Table 1: MACS Analysis Cell Count-1

Fraction ID	Cell Count	% Total
Starting	2.0×10^8	-
LD Elution	8.6×10^6	**4.3%**
LD Fraction	25×10^6	**12.5%**
LS Elution	7.0×10^6	**3.5%**
LS Fraction	0.5×10^6	**0.25%**

Table 1 illustrates the cell counts from the first MACS analysis performed on splenic cells. The starting number of cells for this assay was 2.0×10^8. The separation resulted in the isolation of 0.25% $CD138^+$ cells from the entire population. The LD fraction contains general plasma cells. The LS fraction contains CD 138^+ B cells.

Table 2: MACS Analysis Cell Count-2

Fraction ID	Cell Count	% Total
Starting	1.12×10^8	-
LD Elution	1.07×10^7	**9.6%**
LD Fraction	4.9×10^7	**43.8%**
LS Elution	4.0×10^6	**3.6%**
LS Fraction	0.3×10^6	**0.27%**

Table 2 illustrates the cell counts from the second MACS analysis performed on splenic cells. The starting number of cells for this assay was 1.12×10^8. The separation resulted in the isolation of 0.27% $CD138^+$ cells from the entire population.

A FACS analysis was performed on the MACS purified cells to determine whether the MACS protocol successfully isolated $CD138^+$ plasma cells from the entire population of spleen cells (**Figure-1**). The outcome of the separation was compared with the outcome of the separation performed by Miltenyi Biotec (the same biotech company from which the MACS kit was purchased). Panels A, B, and C are histograms from the separation done by Miltenyi Biotec, while D, E, and F were performed in-house. Panels A and D are histograms from the starting cell fraction, the spleen cells before separation. When comparing A and D, it can be seen that only a portion of these starting cells express $CD138^+$ (a marker for mature B cells) (X-axis), while a significant portion stain for CD45R (B220) (a marker for immature B cells) (Y-axis). In addition, both panels

show a significant portion of cells staining negative for both CD138 and B220, which likely represent non-plasma cells. Panels B and E denote the LD elution fraction containing pre-enriched plasma cells after depletion of B220 immature cells. As expected, these LD elution cells stained negative for B220 (Y-axis), while a small portion stained positive for CD138 (X-axis).

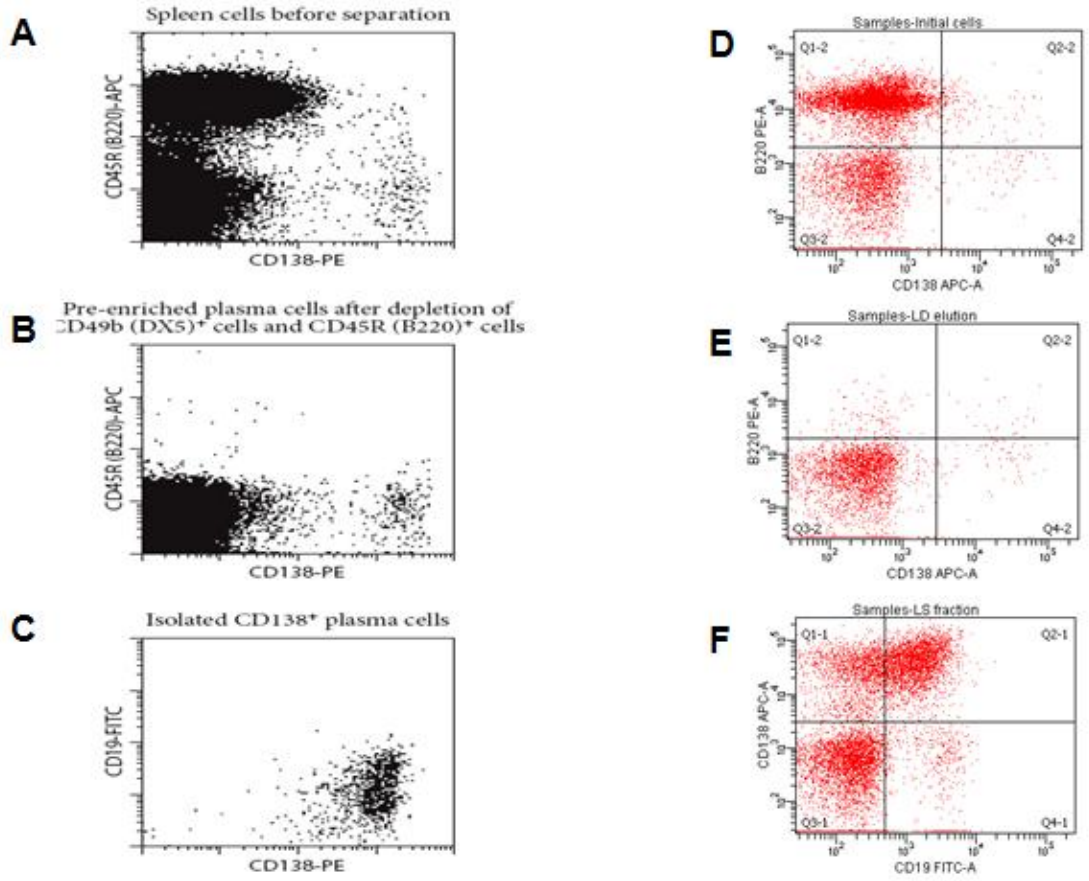

Figure 1: FACS Analysis of the MACS Separations Using the CD138$^+$ Plasma Cell Isolation Kit. The effectiveness of the MACS separation was determined by using FACS analysis on the various fractions. Panels A, B, and C show a FACS analysis performed by Miltenyi Biotec. Panels D, E, and F were performed in-house. In this experiment, CD19 was labeled with FITC, BD220 with PE, and CD138 with APC. The five fractions screened were: Initial Cells, LD elution, LD fraction, LS elution, and LS fraction. Note the change in the axis labels between C and F.

Panels C and F represent cells eluted from the LS column, and theoretically contain mature B cells. The Miltenyi analysis shows these cells stain strongly for CD138

(X-axis) and lack CD19 (Y-axis) (a marker present on immature B cells, but lost when B cells mature into plasma cells). This data conflicts with the in-house analysis showing a mixture of CD138 positive and negative cells (Y-axis), and a mixture of CD19 positive and negative cells (X-axis). This indicates the separation was successful in enriching the $CD138^+$ cells compared to the starting population, but not successful in isolating a pure population of $CD138^+$ cells.

The various fractions from the separations were fused with myeloma cells and cultured. From data shown in **Table 3** and **4,** it can be seen that using MACS to separate $CD138^+$ cells (LS fraction) resulted in a decrease in the number of cells being fused. This decrease in the number of cells being fused led to a reduction in the quantities of materials used for the fusion. In a typical fusion, approximately 1.0 $x10^8$ spleen cells would have been used. After isolating the $CD138^+$ plasma cells, 5.6 $x10^6$ spleen cells were fused with 3.0 $x10^6$ SP2 myeloma cells (Table 3). In fusion-2, 1.0 $x10^7$ $CD138^+$ cells were fused with 5.0 $x10^6$ myeloma cells (Table 4). These numbers of SP2/0 cells were chosen to maintain a 5:1 ratio of spleen cells to myeloma cells. Fusing a population of 1.0 $x10^8$ spleen cells with the SP2 tumor cells would require approximately 1ml of PEG. After the separation, the amount of PEG required reduced to only 50 μl, and 83 μl, for fusion-1 and -2, respectively. In addition, a normal fusion of 1.0 $x10^8$ cells requires approximately 20-30 plates being seeded and screened. By fusing a smaller population of cells, less screening was required as only 5-7 plates were plated. Thus, separating $CD138^+$ B cells from remaining plasma cells before fusion reduces the amount of materials used for the fusion.

Table 3: Materials Used for Fusion-1

Fraction ID	Cell #	Sp2 Cell #	PEG (ul)	SFM
Starting	3.6×10^7	1.8×10^7	200	4 ml
LD Fraction	3.0×10^7	1.5×10^7	200	4ml
LS Elution	3.0×10^7	1.5×10^7	200	4ml
LS Fraction	5.6×10^6	3.0×10^6	50	950 ul

Shown in Table 3 are the amounts of spleen cells, SP2 myeloma cells, PEG, and SFM used for Fusion-1. Four fractions were fused: Starting (plasma and non plasma cells), LD Fraction (pre enriched plasma cells), LS Elution ($CD138^-$ plasma cells), and LS Fraction ($CD138^+$ plasma cells).

Table 4: Materials Used for Fusion-2

Fraction ID	Cell #	Sp2 Cell #	PEG (ul)	SFM (ml)
Starting	3.0×10^7	1.5×10^7	166	4
LS Elution	3.0×10^7	1.5×10^7	166	4
LS Fraction (0.5×10^6)	1.0×10^7	5.0×10^6	83	1
LS Unfused	1.0×10^6	-	-	-

Shown in Table 4 are the amounts of spleen cells, Sp2 cells, PEG, and SFM used for Fusion 2. 5 fractions were fused: Starting (plasma and non plasma cells), LS Elution ($CD138^-$ plasma cells) LS Fraction ($CD138^-$ plasma cells), and LS Unfused ($CD138^+$ plasma cells that were not fused with sp2 cells).

After the fusion procedure, the hybridomas were incubated at 37^oC with 5% CO_2 for two weeks. During this incubation period, macroscopic cell colony growth was monitored at various points. The amount of growth in each fraction as well as the concentration resulting in optimal growth was observed. The cell growth for Fusion-1 is shown in **Table 5**. Seven days post fusion, 288 wells were screened in the fraction labeled "Starting". Of the 288 wells, only 37 (12.8%) contained macroscopic colonies. However, 14 days post fusion, 150 colonies were observed in 288 wells (52.0%). In the LS Fraction (5×10^5), 95 out of 96 (98.9%) screened wells contained colonies 14 days post fusion. Interestingly, in the LS Fraction (5×10^4) plate, only 24 out of 96 wells (23.9%) contained colonies. While most of the fractions experienced a dramatic increase

in the number of observable colonies between days 7 and 14, this increase was not seen in the LS Fraction (5×10^4) plate.

Table 5: Colony Growth of Fused Cells from Fusion-1

Fraction ID	Number of Colonies (Day 7) + growth in wells / total wells plated	Number of Colonies (Day 14) + growth in wells / total wells plated
Starting	37/288 (12.8%)	150/288 (52.0%)
LD Elution	32/288 (11.1%)	110/288 (38.2%)
LS Elution	11/288 (3.8%)	136/288 (47.2%)
LS Fraction (5×10^5)	73/96 (76.0%)	95/96 (98.9%)
LS Fraction (5×10^4)	23/96 (23.9%)	24/96 (25.0%)

Fractions were seeded in 96 well plates at various densities of spleen cells per well, and incubated at 37°C. The growth of macroscopic colonies was monitored 8 and 15 days post fusion.

Table 6 shows the various fractions plated for Fusion-2. In the "Starting" fraction, 26 colonies were observed out of 288 plated wells (9.0%) at 14 days post fusion, which is substantially below the 52% observed for Fusion-1. In the LS elution plate (containing non-B cells), 0 colonies were observed even after 14 days. As a control experiment, unfused cells were plated to confirm the hypothesis that plama cells would not survive in the absence of fusion. As expected, no colonies were observed in the plate with unfused cells. Very few living cells could be found in this fraction. In addition, five concentrations of the LS Fraction were plated to determine the concentration. that resulted in optimal hybridoma growth. In the LS Fraction (0.5×10^6), 0 colonies were observed 14 days post fusion (**Table 6**), which is substantially lower than observed for Fusion-1 with the same number of cells. However, plating the cells in concentrations of 1×10^5 and 2×10^4 resulted in 7 and 4 colonies, respectively. Plating the cells at concentrations lower than 2×10^4 resulted in no colony growth.

Table 6: Colony Growth of Fused Cells from Fusion-2

Fraction ID	Number of Colonies (Day 14) + growth in wells / total wells plated
Starting	26/288 (9.0%)
LS Elution	0/288
LS Unfused	0/96
LS Fraction ($0.5x10^6$)	0/96
LS Fraction ($1 x10^5$)	7/96
LS Fraction ($2x10^4$)	4/96
LS Fraction ($4 x10^3$)	0/96
LS Fraction ($8 x10^2$)	0/96

Fractions were seeded in 96 well plates, at a various densities of spleen cells per well, and incubated at 37°C. The growth of macroscopic colonies was observed 14 days post fusion. Plating of the unfused cells as well as plating cells with low density (10^3 and10^2) resulted in no growth.

To determine the fused cells' abilities to produce antibodies, an IgG ELISA was performed on supernatants from both fusions (**Table 7**). Only two fractions were tested for this experiment. Out of 95 tested wells in the "Starting" fraction, only 9 wells (9.4%) contained IgG-producing hybridomas, while 8/23 wells (34.7%) of the LS fraction ($5x10^4$ cells) contained IgG-producing hybridomas.

Table 7: ELISA Screening for IgG Producing and Antigen Reactive Hybridomas

Fraction ID	IgG Producing	Human Antigen Reactive	mAb Candidates
Starting	9/95 (9.4%)	4/95 (4.2%)	4/9 (44.4%)
LD Elution	-	8/95 (8.4%)	-
LS Elution	-	0/95 (0.0%)	-
LS Fraction-$5x10^5$	-	38/95 (40.0%)	-
LS Fraction-$5x10^4$ (24 wells)	8/23 (34.7%)	5/23 (21.7%)	5/8 (62.5%)

Table 7 shows the results from the IgG ELISA and the human antigen specific ELISA. Hybridomas from Fusion-1 were used for this assay. 9 hybridomas were IgG producing and 4 were antigen reactive in the Starting fraction. The LS Fraction with $5x10^4$ cells/well plate contained 8 IgG producing and 5 antigen reactive hybridomas. Determination of positive and negative hybridomas was done by comparing the OD450 of the positive control to the remaining wells.

Because it was not known whether the hybridomas were producing IgG, or if the unfused spleen cells had produced IgG during the two week incubation period, 23 colonies from the LS Fraction-$5x10^4$ were selected and transferred to a new plate and cultured. Out of the 23 screened wells, 8 were IgG producing hybridomas. This confirmed that the hybridomas were producing the IgG and not the unfused spleen cells.

Experiments were also designed and performed to test the antibody's ability to react with a specific antigen. For Fusion-1, the mice were immunized with human IgG as a general immunogen to test mouse antibody formation against a human protein. The hybridoma antibodies abilities to react to human IgG was tested using an ELISA. The results of this experiment are shown in **Table 7**, third column. In the Starting plate, 4 out of 95 tested wells (4.2%) contained antibody-producing cells that reacted with human IgG. The LD Elution fraction contained 8 antigen reactive wells out of 95 (8.4%) screened wells. This increase in number was expected as the LD Elution Fraction contained pre-enriched plasma cells. No antigen-reactive colonies were observed in the LS elution fraction, containing predominantly $CD138^-$ plasma cells. Both concentrations of the LS Fractions contained antigen reactive colonies. In LS Fraction-$5x10^5$, approximately 40% of the cells were reactive to the human antigen. This increase in reactivity was expected as the LS Fraction contained only mature B cells and the chances of obtaining an antigen specific hybridoma were higher. Hybridomas were labeled as "positive" for IgG production and antigen reactivity if their OD_{450} was similar to that of the positive control. **Figures 2** and **3** are examples of plates that were screened using ELISAs.

Figure 2: ELISA Screening for IgG Producing Hybridomas

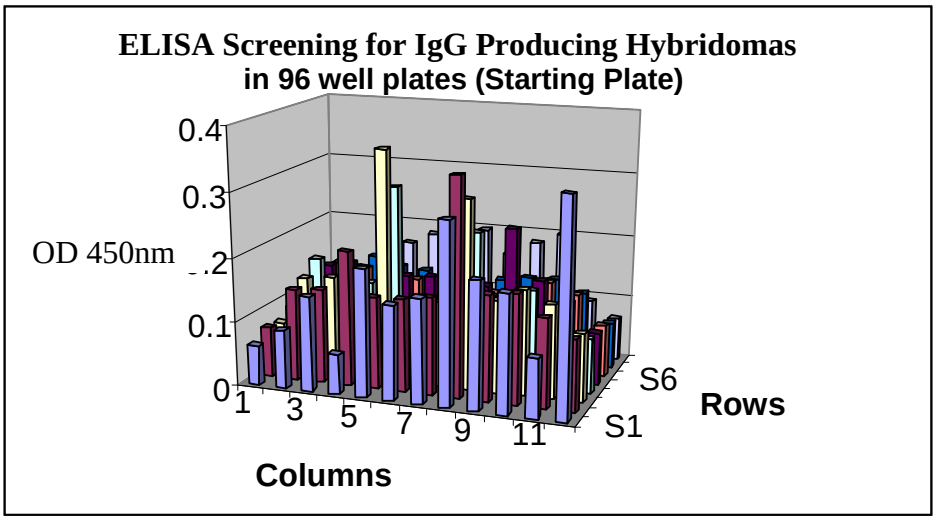

Figure 2 shows the results from the IgG ELISA performed on hybridomas from Fusion-1. The Starting fraction is shown in this figure. Positive cells were determined by comparing the OD_{450} of the positive control (row S1 column-12) to the remaining wells. The positive control had an OD_{450} of approximately 0.300. In this specific ELISA, wells with OD_{450} above 0.200 were considered to be potential antibody producing hybridoma candidates.

Figure 3: ELISA Screening for Antigen Reactive Hybridomas

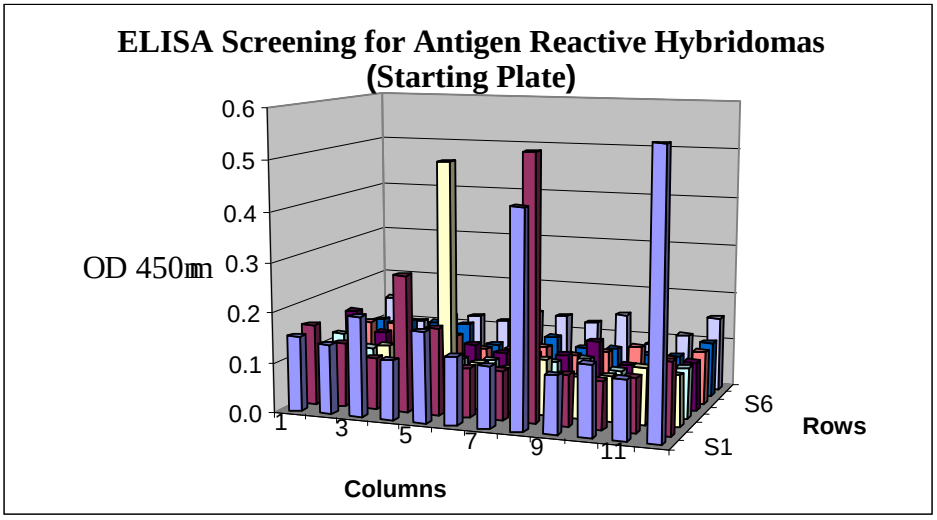

Figure 3 shows the results from the ELISA used to screen for antigen reactive hybridomas. The Starting fraction from Fusion-1 is shown in this figure. Positive cells were determined by comparing the OD_{450} of the positive control (row S1 column12) to the remaining wells. In this case, the positive control had an OD_{450} of approximately 0.500. Wells with OD_{450} above 0.290 were considered to be potential antigen reactive hybridoma candidates.

Similar experiments as mentioned above were also performed for Fusion-2 (**Table 8**). For the second fusion, mice were immunized with 293 cells pre-transfected with RON Delta 160 antigen. Unlike the first fusion, this fusion was not as successful in producing antigen-specific hybridomas, and growth was slow. All fractions from Fusion-2 were screened using ELISAs for undesirable mouse IgG Production. The Starting plate and the LS Fraction ($0.5x10^6$) plate showed that approximately 50% of the screened wells contained undesirable mouse IgG-producing hybridomas, indicating that in this fusion-2 experiment pre-selecting for $CD138^+$ mature B cells did not increase the percent of successful hybridoma production. In the LS Fraction ($1\ x10^5$) plate (containing $CD138^+$ cells), only 7 out of the 95 (7.3%) screened wells were undesirable mouse IgG-producing hybridomas. However, once again, it was not clear whether this was a result of the hybridoma producing the IgG, or if the spleen cells had produced IgG during the two week incubation period. In addition to examining undesirable mouse IgG production, the hybridoma's ability to react to the RON Delta 160, the antigen the mice vaccinated with, was tested using FACS on BaF3 cells expressing the antigen on the surface (Table 8, third column). In this experiment, unfortunately the fractions did not contain any antigen-reactive hybridomas.

Table 8: ELISA and FACS Screening for IgG-Producing and Antigen-Specific Hybridomas

Fraction ID	IgG Producing (ELISA)	Antigen Reactive (FACS)	Antibody Candidates
Starting	51/95 (53.6%)	0/95	0/95
LS Unfused Cells	1/95 (1.0%)	0/95	0/95
LS Elution	7/95 (7.3%)	0/95	0/95
LS Fraction ($0.5x10^6$)	53/95 (55.7%)	0/95	0/95
LS Fraction ($1 x10^5$)	7/95 (7.3%)	0/95	0/95

Table 8 shows the results from the IgG ELISA and the 293 RON Delta 160 FACS analysis on BaF3 cells. Hybridomas from Fusion 2 were used for this assay. In this ELISA, every plate had at least one IgG producing hybridoma. The Starting fraction and the LS Fraction ($0.5x10^6$) showed approximately 50% of screened wells contain IgG producing hybridomas. In the LS Fraction ($1 x10^5$), only 7 out of the 95 screened wells were IgG producing hybridomas. IgG positive hybridomas were determined by comparing the OD_{450} of the positive control to the remaining wells. None of the IgG producing hybridomas were antigen reactive when analyzed using FACS. Antigen reactive hybridomas were determined by comparing the FL2GeoMeans of the positive control to the remaining wells.

DISCUSSION

The objective of this study was to improve the efficiency of monoclonal antibody production by pre-isolating a subpopulation of antibody producing plasma cells prior to fusion using magnetic activated cell sorting. While the data from Fusion-1 and Fusion-2 are not consistent as to whether using MACS to separate the $CD138^+$ cells results in an increase in the number of antigen-specific hybridomas, it is clear that this technique has both its advantages and disadvantages.

Using MACS to separate $CD138^+$ B cells was shown in this project to decrease the total number of cells required for fusion, and decrease the quantities of materials needed for the fusion. In addition, it is less tedious and more cost efficient to screen only 3 plates to obtain 5 possible hybridoma candidates for scale up and mass production, as opposed to screening 60 plates and obtaining only 10 possible hybridoma candidates for antibody production. Thus, regardless of whether the purification process alters the percent of fused cells, the MACS $CD138^+$ procedure reduces reagent usage, and greatly decreases the number of plates required for screening.

However, this procedure does have its disadvantages. As seen from the FACS analysis data, the $CD138^+$ Plasma Cell Isolation Kit did not successfully select for only $CD138^+$ cells based on the in-house FACS analysis. A significant portion of cells expressing CD19 (an immature B cell marker) were also included in the final isolated population (Figure 1). Since CD19 is also a B cells marker, it was expected that a few cells expressing low levels CD19 would also be present in the purified cell fraction. In addition, cells expressing B220 (another marker for immature B cells) were also present in the final fraction (data not shown). However, the isolation kit did successfully enrich

the population of cells expressing CD138. The difference in results of $CD138^+$ isolated fraction may be due to a change in the MACS protocol; while the isolation kit by Miltenyi Biotec called for an MS column to isolate the $CD138^+$ cells, an LS column was used instead in this study. The smaller MS column may have been more precise in selecting for $CD138^+$ cells.

The results from the experiments also suggest that the fusion of the $CD138^+$ cells with myeloma cells does not always produce antigen reactive hybridomas. While experiments performed on supernatants from Fusion-1 provided promising results, this was not the case for Fusion-2. From the time of the original Kohler and Milstein's study, it has been proven that each fusion is unique in that it can either produce many mAb candidates or can produce no candidates. More repeats of these experiments need to be done to determine if the process of separating the $CD138^+$ cells prior to fusion is worthwhile. Further characterization of the hybridomas also needs to be done, including determining their isotypes, affinities, and epitopes. In addition, a replication of this experiment can also be performed on cells from bone marrow and lymph nodes to determine if the type of tissue being fused has an effect on cell growth, IgG production, and antigen reactivity.

After performing two separations and two fusions, it was concluded that although this technique of separating the B cells before fusion does result in fewer number of cells being fused, it does not necessarily result in an increased number of IgG producing or antigen-reactive hybridomas.

BIBLIOGRAPHY

1. Alkan, S. "Monoclonal antibodies, the story of a discovery that revolutionzied science and medicine." Nature (2004): 153-156.

2. Brody T. "Identification of two cell populations required for mouse immunocompetence." Journal of Immunology 105 (1970): 126-138.

3. De Wildt RM, Steenbakkers PG, Pennings AH, Van Den Hoogen FH, Van Venrooij WJ, Hoet RM. "A new method for analysis and production of monoclonal antibody fragments originating from single human B cells." Journal of Immunological Methods 207 (1997): 61–67.

4. Egeland T, Hovdenes A, Lea T. "Positive selection of antigen specific B lymphocytes by means of immunomagnetic particles." Scandinavian Journal of Immunology 27 (1988): 439–444.

5. Hoven MY, De Leij L, Keij JF. "The TH. Detection and isolation of antigen-specific B cells by the fluorescence activated cell sorter (FACS)." Journal of Immunological Methods 117 (1989): 275-284.

6. Irsch J, Hunzelmann N, Tesch H et al. "Isolation and characterization of allergen-binding cells from normal and allergic donors." Immunotechnology 1 (1995): 115–125.

7. Julius MH, Masuda T, Herzenberg LA. "Demonstration that antigen binding cells are precursors of antibody producing cells after purification with a Fluorescence Activated Cell Sorter." Proceedings of the National Academy of Sciences 69 (1972): 1934–1938.

8. Kodituwakku A, Jessup C, Zola H, and Don Roberton. "Isolation of antigen-specific B cells." Immunology and Cell Biology 81 (2003): 163-170.

9. Kohler, G and C Milstein. "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity." Nature (1975): 495-497.

10. Leyendeckers H, Odendahl M, Lohndorf A et al. "Correlation analysis between frequencies of circulating antigen-specific IgG bearing memory B cells and serum titers of antigen-specific IgG." European Journal of Immunology 29 (1999): 1406–1417.

11. Margulies, D. "Monoclonal antibodies: Producing Magic Bullets by Somatic Cell Hybridization." Pillars of Immunology (2005): 2451-2452.

12. Mcheyzer-Williams LJ, Cool M, Mcheyzer-Williams MG. "Antigen-specific B cell memory: expression and replenishment of a novel b220-memory B cell compartment." Journal of Experimental Medicine 191 (2000): 1149-11466.

13. Napaporn A, Phunpae P, Watchara K. "A modified hybridoma technique for production of monoclonal antibodies having desired isotypes." Cytotechnology 60 (2009): 45-51.

14. Reichert, Janice and Clark Rosenweig. "Monoclonal antibody successes in the clinic." Nature Biotechnology (2005): 1073-1078.

15. Steenbakkers PG, Van Wezenbeek PM, Plijve W. "Immortalization of antigen selected B cells." Journal of Immunological Methods 163 (1993): 33-40.

16. Walker SM, Meinke GC, Weigle WO. "Enrichment of antigenspecific B lymphocytes by the direct removal of B cells not bearing specificity for the antigen." The Journal of Experimental Medicine 146 (1977): 445–456.

17. Wigzell H, Anderson B. "Cell separation on antigen-coated columns." The Journal of Experimental Medicine 129 (1968): 23-36.