PREPARATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST THE NATURAL KILLER CELL GLYCOLIPID ASIALO-GM1

by

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Preparation of Monoclonal Antibodies Directed Against the Natural Killer Cell Glycolipid Asialo-GM1

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ABSTRACT

Natural killer (NK) cells are a subpopulation of large, granular lymphocytes that are able to lyse various types of tumor and virus infected cells without prior sensitization. Because of their tumor surveillance characteristics, NK cells have been the subject of many studies in humans and mouse systems. One problem with studying NK cells is that they have shared surface molecules with other cells which makes it difficult to identify them. Several years ago it was discovered that mouse NK cells express the cell surface molecule ganglio-N-tetraosylceramide (asialo-GM1). The molecule is expressed primarily on NK cells and is thought to play a role in their cytolytic activities. Currently, commercially available polyclonal antibodies against asialo-GM1 are used for in vivo and in vitro studies to identify NK cells. Unfortunately these antibodies are nonspecific and thus cross react with other molecules. To bypass this problem we utilized the hybridoma technology to make monoclonal antibodies directed against asialo-GM1 which should be more specific and less cross reactive. In this study we immunized NZW mice with asialo-GM1 and fused its spleen cells with a tumor cell to make a hybrid cells. From these hybrids, five clones which are secreting antibody against asialo-GM1 were isolated. The products of these hybrids will be used as a research tool for further studies on natural killer cells.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Diana Fagan, for her guidance, suggestions and encouragement throughout the preparation of this thesis.

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Finally, I would like to thank my family for their patience and support while I worked on this project.

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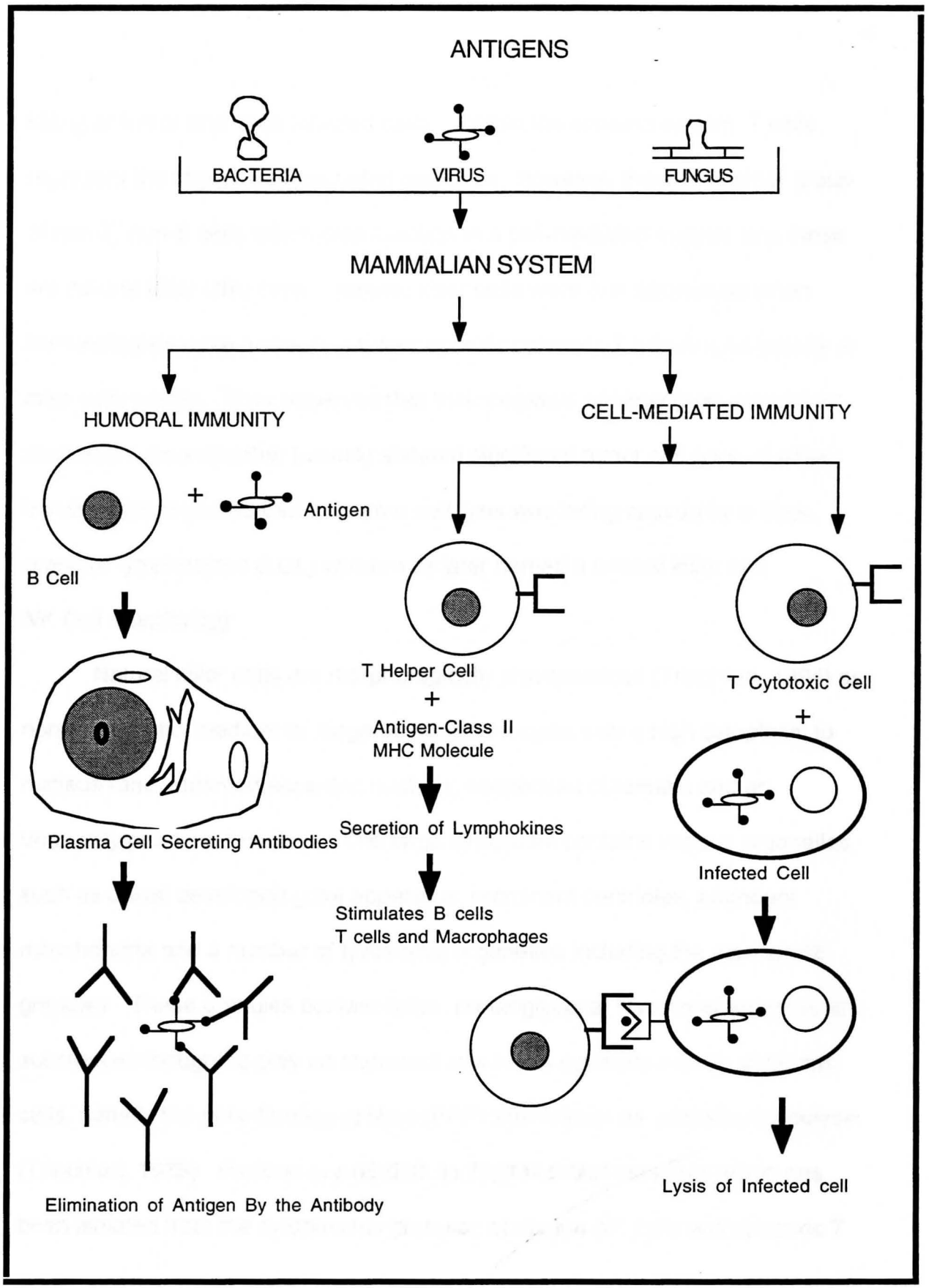
ABBREVIATIONS

Ab	Antibody
Ag	Antigen
Asialo-GM1	Asialoganglioside GM1
CD	Cluster of Differentiation
ELISA	Enzyme-Linked Immunosorbent Assay
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
IL-2	Interleukin-2
ICAM	Integrin Cell Adhesion Molecule
mAb	Monoclonal Antibody
MHC	Major Histocompatability Complex
NK Cell	Natural Killer Cell
NKCF	Natural Killer Cell Cytotoxic Factor
NZW	New Zealand White (mice)
PBL	Peripheral Blood Lymphocyte
PFP	Pore-forming Protein

INTRODUCTION

The ability of the body to fight off foreign invaders and the various associated immunological mechanisms have been the subject of numerous studies since the English physician Edward Jenner discovered in 1796 that people developed immunity to smallpox when injected with cowpox. For the last two hundred years significant advances have been made in our understanding of the immunological mechanisms of the immune system. For instance, we now know that individuals developed immunity to smallpox when given cowpox because they were secreting antibodies, such that subsequent exposures resulted in an immediate elimination of the small pox. The most significant outcome of Jenner's discovery, was that it became possible to make vaccines against various pathogens which protect the body from any future exposures to that pathogen. The type of immune response that a vaccine represents belongs to the acquired, humoral branch of the immune response (Figure 1). A humoral response involves the interaction of B cells with antigens (foreign invaders) and their differentiation into antibody-secreting plasma and memory cells. The secreted antibody binds to the antigen and facilitates its clearance from the body, while the memory cells provides a rapid response upon subsequent exposure to the same antigen. The other branch of the immune response is the cell-mediated response. The cell- mediated response involves various subpopulations of T cells that recognize an antigen presented on self cells. T helper cells respond to an antigen by producing cytokines and T cytotoxic cells respond to antigens by developing into cytotoxic T lymphocytes which mediate

Figure 1. A schematic overview of the humoral and cell-mediated branches of the immune system.


ANTIGENS
BACTERIA
VIRUS
FUNGUS
MAMMALIAN SYSTEM
HUMORAL IMMUNITY
CELL-MEDIATED IMMUNITY
+
Antigen
B Cell
T Helper Cell
+
Antigen-Class II
MHC Molecule
T Cytotoxic Cell
+
Plasma Cell Secreting Antibodies
Secretion of Lymphokines
Infected Cell
Stimulates B cells
T cells and Macrophages
Lysis of Infected cell
Elimination of Antigen By the Antibody

killing of tumor and virus infected cells. Within the immune system, T cells represent the classic cell-mediated response; however, there is another group of non-T, non-B cells which also function in a cell-mediated manner and these are natural killer (NK) cells. Natural killer cells were first discovered when immunologists were measuring tumor specific cytotoxic T lymphocyte activity in mice with tumors. They observed that their negative controls (non-immunized mice and mice with other tumors) showed significant tumor cell lysis. Further investigation revealed that the tumor cell lysis was being caused by a large, granular lymphocytes (LGL) which was later named a natural killer cell.

NK Cell Morphology

Natural killer cells are morphologically characterized (Trinchieri, 1994) as non-phagocytic, medium to large-sized lymphocytes with a high cytoplasm to nucleus ratio, indented eccentric nucleus, condensed chromatin and an unusually prominent nucleoli. The large cytoplasm contains various organelles, such as a well developed golgi apparatus, prominent centrioles, abundant mitochondria and a number of lysosomal organelles including the azurophilic granules. These granules contain lipids, proteoglycans, lysosomal enzymes and substances thought to play an important role in the cytotoxic activity of the NK cells, namely the pore-forming protein (PFP) also known as perforin or cytolysin (Trinchieri, 1989). Perforin is a 65,000 to 70,000 dalton molecule which has been isolated from the cytoplasmic granules of murine NK cells and cytotoxic T

lymphocytes. The structure of perforin from mice, humans and rats have been shown to consist of 534 amino acids with a 60,000 dalton core polypeptide. The perforin isolated from the three species exhibit the following homology: murine and human (68%); human and rat (69%); and mouse and rat (85%) (Yagita et. al., 1992). This molecule also has a 20 % homology to the complement component MAC (Membrane Attack Complex) which is known to cause holes in the membrane of target cells, a function similar to that of perforin.

NK cell Surface Markers

Cell surface markers are molecules which are expressed by certain lymphocytes based on their lineages or maturational stage. The nomenclature for these markers were established after immunologists (worldwide) produced monoclonal antibodies against specific surface molecules on B and T cells, macrophages, neutrophils and NK cells. Infrequently, international workshops were held and the specificities of each antibody were compared. Where a cluster of monoclonals were found to react with the same polypeptide, they were then grouped together as a cluster of differentiation (CD). The CD nomenclature was originally developed for human leukocyte membrane molecules; however, homologous molecules in other species, such as mice are commonly referred to by the same CD designation.

Extensive studies on the surface phenotype of natural killer cells have demonstrated that they have shared myelomonocytic and T cell related markers (Table 1). Human NK cells are phenotypically characterized as $CD3^-$, $CD16^+$,

$CD8^+$, $CD7^+$, Leu 7^+ (a cell surface marker, lacking a CD designation), $CD4^-$, $CD56^+$ and $CD11^+$. The surface antigen CD11 is shared with both monocytes and granulocytes while CD16 is shared with all granulocytes. Of the remaining surface markers, all except CD16 and CD56 are found on T lymphocytes. Murine NK cells are characterized as $CD11^+$, NK 1.1^+, Asialo- $GM1^+$, Thy-1^+, NK 2.1^+, Qa-2^+ and Qa-5^+. However, no one phenotype is found on all murine NK cells because they are for the most part a phenotypically heterogenous population of cells. All of these surface markers with the exception of NK 1.1 and 2.1 and asialo-GM1 are expressed by T lymphocytes. In addition, CD11 is expressed by both monocytes and granulocytes. Asialo-GM1 which is expressed primarily on murine NK cells is also found on macrophages. Since NK cells and T lymphocytes have similar cytolytic activity, it is possible that the shared surface markers play a role in their lytic function. Additionally, the shared markers of NK and T lymphocytes supports the hypothesis that NK cells are closely related to T lymphocytes.

NK cell Ontogeny

Within the cell-mediated branch of the immune response, NK cell represent a unique lineage of lymphocytes which are characterized by their ability to lyse tumor cells, virus infected cells and some normal target cells without the requirement of a major histocompatibility complex (MHC) restriction for antigen recognition (Hogan and Basten, 1988). Several studies have

Table 1
Partial List of Cell Surface Molecules

Surface Molecules	Cellular Association	Function
CD3	T_c, T_h	Signal transduction molecule
CD4	T_h, Mϕ	Adhesion molecule that binds class II MHC molecule
CD7	T, NK	?
CD8	T_c, NK	Adhesion molecule that binds class I MHC molecules
CD11	B, T, NK	Accessory molecules that binds ICAM 1
CD16 (FcγRIII)	NK, Mϕ, Gr	Low affinity receptor for the Fc region of IgG.
CD56	NK	Adhesion molecule; Used to purify human NK cells.
Leu 7	T, NK	Associated with cortical thymocytes
NK1.1	NK	NK marker; found on certain strains of mice NK cells.
NK2.1	NK	NK marker; found on certain strains of mice NK cells.
Qa-2	T, NK	Non-classical MHC associated class I molecule of mice
Qa-5	T, NK	Non-classical MHC associated class I molecule of mice
Thy-1	T, NK	Mouse Signal transduction molecule
Asialo-GM1	NK, Mϕ	Mediates cytotoxic activity of mouse NK cells.

A partial list of cell surface molecules associated with NK cell as well as other cells. Abbreviations: B=B cells, T=T cells, NK=Natural killer cells, Mϕ =Macrophage, Gr = Granulocytes, T_c=Cytotoxic T cell, T_h=Helper T cell, MHC=major histocompatability complex.

demonstrated that NK-like cells can be found in various invertebrates such as annelids, mollusks, arthropods and echinoderms. They have also been isolated from vertebrates such as dogs, cats, swines, rodents, birds and humans (Savary and Lotzova, 1986). The discussions in this paper will focus primarily on humans and rodents. In experimental studies, there is evidence that NK cells are derived from precursor cells in the bone marrow and they do not require the presence of a thymus for maturation (required for T cell maturation). Rodewald et. al. described a murine population of early fetal thymocytes (immature T cells) with the potential to differentiate towards T or NK cells, depending on the environment. They isolated 14.5 day thymocytes which were $CD4^-$, $CD8^-$, $Fc\gamma RII/III^+$ and cultured them in thymic (thymus cells) and non-thymic environment. (CD4 is a surface molecule which is found on T helper cells and plays a role in signal transduction as well as acting as adhesion molecules that bind to class I MHC molecules. CD8 is also a surface molecule which is found on T cytotoxic cells and binds to class II MHC molecules. FcγRII/III is a low affinity receptor for the Fc region of the immunoglobulin IgG. It is found on natural killer cells as well as B cells, macrophage and granulocytes which allows them to perform antibody dependent cellular cytotoxicity). The cells which were cultured in the thymic environment developed into T cells and the cells cultured in the non-thymic environment developed into NK cells. This suggested that precursors of both T and NK cells may be derived from a phenotypically homogenous subset of thymocytes.

The distribution of NK cells is limited, with NK cells identified primarily in the lymph node, spleen, liver and peripheral blood of non-immunized rodents and normal humans (Lanier et. al., 1986). Morphological studies have shown that NK cells makeup 3.6% of the peripheral blood lymphocytes (PBL) of normal individuals. However, studies with monoclonal antibodies [CD16, NKH-1: human natural killer cell antigens] have shown that they actually constitute 15% of the PBL with a large variability among individuals. The spleen is the major source of NK cells in both human and other animals, with approximately 3-4% of all white cells within the red pulp region being NK cells (O'Shea and Ortaldo, 1992; Trinchiera et.al., 1989).

NK Cell Cytolytic Activity

Natural killer cells are the "first line of defense" against tumor cells and virus infected cells which they lyse spontaneously. Unlike cytotoxic T cells, NK cells lyse target cells without any restriction imposed by the major histo-compatibility complex (MHC). The major histocompatibility complex is a genetic region on chromosome 6 in humans and 17 in mice, encoding molecules involved in antigen presentation to T cells. The MHC is referred to as the HLA complex in humans and H-2 complex in mice. Three classes of molecules are encoded by the MHC genes, class I genes encode glycoproteins expressed on the surface of all nucleated cells, where they present the antigens of altered self-cells to activate the T cytotoxic cells. Class II genes encode glycoproteins expressed primarily on antigen presenting cells such as macrophages, dendritic

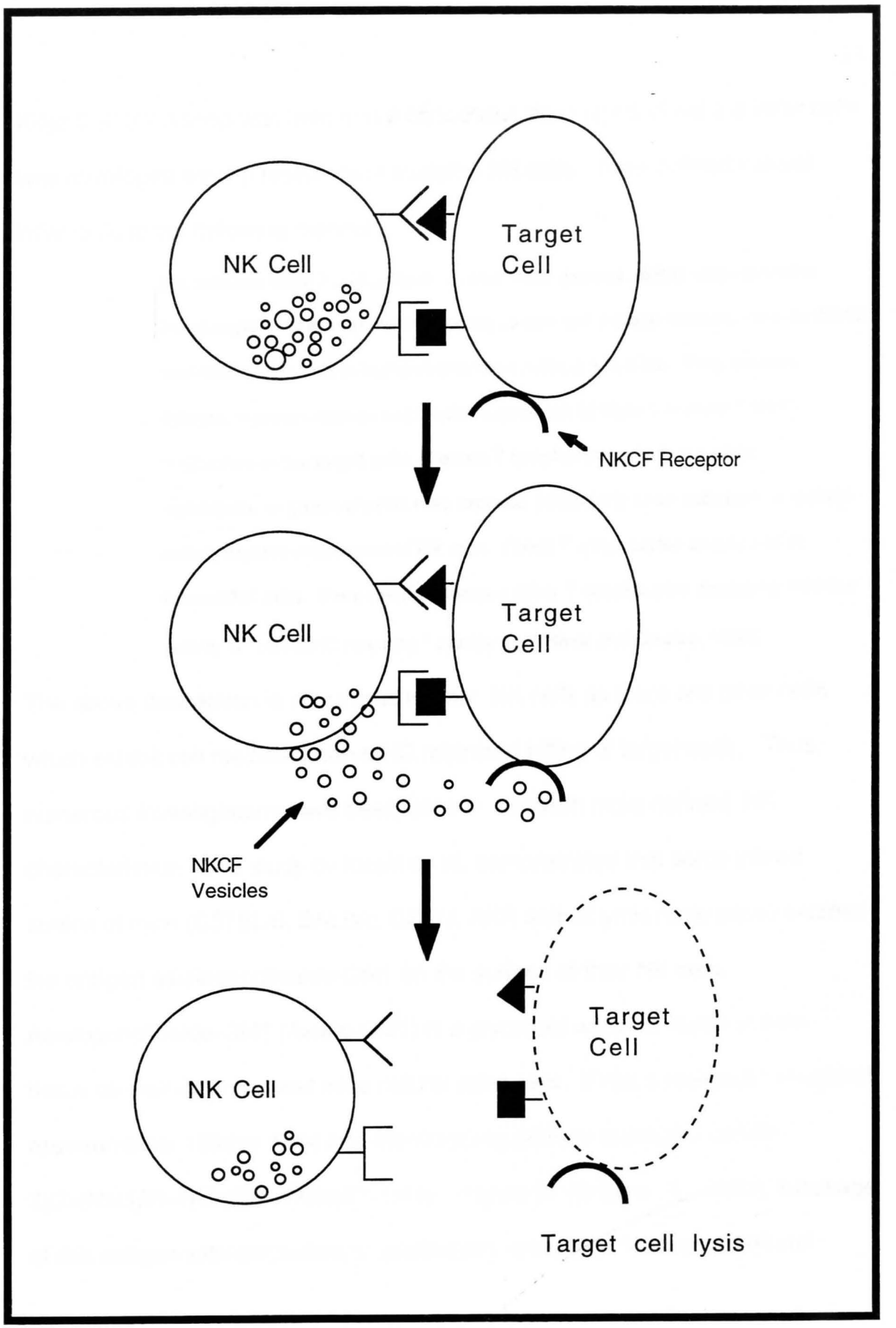
cells and B cells which present antigens to T helper cells. Class III genes encode various proteins such as tumor necrosis factor which are involved in the immune response. "MHC restriction" is the requirement that T cells recognize processed antigen only when presented by MHC molecules of the class associated with the T cell. As a rule, T helper cells recognize antigens presented in the context of a class II MHC molecule and T cytotoxic cells recognize antigens associated with a class I MHC molecule. Interestingly, in contrast to its role in T cell activation, MHC molecules seem to have a negative effect on the activity of NK cells. It seems that tumor cells which express MHC class I molecules inhibit the ability of NK cells to lyse them. NK cells have been shown to have receptors (p58:human NK cell; Ly49:murine NK cells) that recognize MHC molecules, resulting in the inhibition of tumor cell lysis (Moretta et. al., 1994). Transformation or virus infection of many cells causes a decrease expression of self MHC molecules. The decreased levels of MHC molecules on these cells makes them susceptible to NK cell cytolysis.

NK cell mediated lysis of tumor or virus infected cells follows a programmed series of steps (Figure 2). The first stage involves the interaction of a receptor on the NK cell with a ligand on the target cell membrane. These ligands are generally carbohydrates found in high abundance on tumor cells. In the process of binding, the NK cells receive signal from the target cell which activates the natural killer cytotoxic factor (NKCF) release mechanism. Biochemical analysis on NKCF have shown that it has a molecular weight of 15-

30 Kdaltons and a disulfide bond which contributes to a conformation which is responsible for its lytic activity (Bonavida and Wright, 1986). The activation of NKCF release is thought to occur through a second receptor activation process. It has been shown through activation with tetraphorbol acetate and ionophores (non-specific cell activators) that binding of the second receptors result in the metabolism of the membrane lipid phosphotidylinositol biphosphate to generate inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG). This leads to the release of calcium into the cytosol from the endoplasmic reticulum. The accumulation of calcium in the cytosol causes protein kinase C to bind to the cytoplasmic side of the plasma membrane so that DAG can activate protein kinase C. The activated protein kinase C phosphorylate the NKCF protein and activate it (Bonavida and Wright, 1986). Release of the activated NKCF occurs in close proximity to the target cells. However, target cell sensitivity to lysis by NK cells is dependent on the presence of an NKCF binding site on the target cells which serves as a receptor for absorption of the soluble mediator (NKCF). Once the NKCF binds to the target cells it is internalized and the cell is lysed while the NK cell is recycled to lyse another target cell. In addition to the release of NKCF, pore-forming proteins are also released. These molecules bind to the target cell membrane and form pores in the membrane. A combination of the pores and the NKCF contribute to the lytic effect of natural killer cells.

Since NK cells are not the only cytotoxic lymphocytes, in 1989 a Natural

Figure 2. Schematic diagram illustrating the steps in the NK cell-mediated lysis of a target cell. The triangular receptor interaction represents the primary receptor and the square receptor interaction represents the secondary receptor. (Note that unlike traditional secretory mechanisms, NK cells release their granules.)


NK Cell
Target Cell
NKCF Receptor
NK Cell
Target Cell
NKCF Vesicles
NK Cell
Target Cell
Target cell lysis

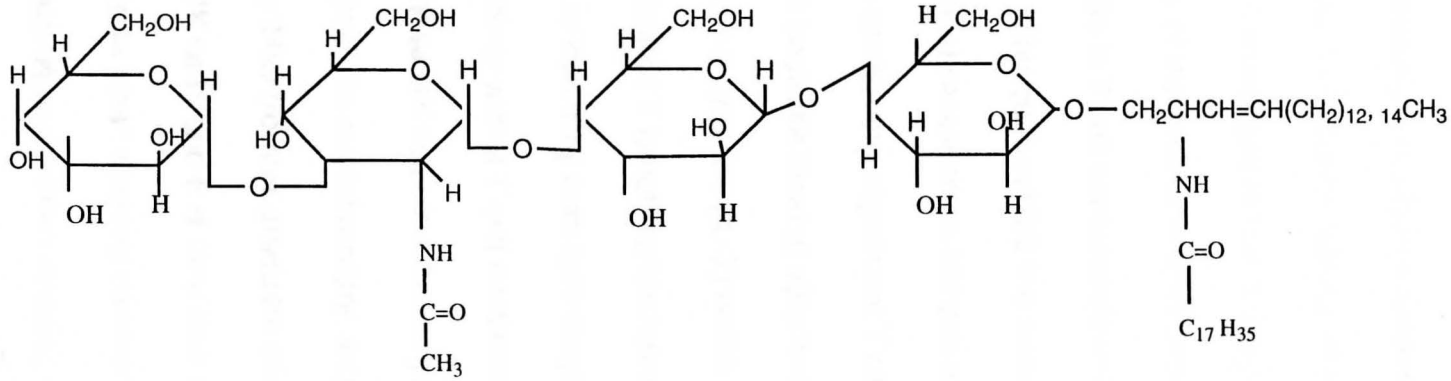
Killer Cell Workshop was held and a consensus description of natural killer cells was developed among researchers studying NK cells. They defined natural killer cells in the following manner:

> NK cells are $CD3^-$ T cell receptor (alpha, beta, gamma, delta)$^-$ large granular lymphocytes. They commonly express certain cell surface markers such as CD16 and NKH-1 (Leu-19) in humans and NK-1.1/NK-2.1 in mice. They mediate cytolytic reactions that do not require expression of class 1 or class 2 MHC molecules on the target cells. Certain T lymphocytes which are either alpha/beta$^+$ or gamma/delta$^+$ may express, particularly upon activation, a cytolytic activity that resembles that of NK cells. These T lymphocytes should not be termed NK cells. They could be termed either T lymphocytes displaying "NK-like" activity or "non-MHC requiring " cytolysis (O'Shea and Ortaldo, 1992).

The above description is not unique to only NK cells as there are other cells which exhibit cell mediated non-MHC restricted killing of target cells. Thus, numerous investigations have been done to establish more defined NK characteristics. One study by Kasai et. al. demonstrated that some inbred strains of mice (C57BL/6, BALB/c, CBA/J, AKR and athymic nude mice) express the antigen asialoganglioside-GM1 on the surface of their NK cells. Asialoganglioside-GM1 (Asialo-GM1) is a glycolipid which is found in brain tissue as well as on rat and mice natural killer cells. It has a molecular weight of approximately 1282 to 1254 and the following general structure: *Gal*(*B*1-3)GalNac(*B*1-4)Gal(*B*1-4)Glc(*B*1-1)Cer (Figure 3), (Arita et. al., 1983). Blockage of this antigen with antibodies to asialo-GM1 resulted in the loss of NK cell

Figure 3. Chemical structure of the molecule Asialoganglioside GM1 which was the antigen used to immunize the mouse in our studies.

ASIALO-GM1



cytotoxic function.

The objective of this study is to make a monoclonal antibody against the surface marker asialo-GM1 which is found on murine natural killer cells. This monoclonal antibody will be used as an investigative tool to identify NK cells in a heterogenous population of cells, One of the problems encountered when studying the activity of natural killer cells is T cell contamination of the NK cell preparation. As stated earlier, natural killer cells exhibit the same cytolytic activity as T lymphocytes. Currently, most research on NK cell activity has been done with human NK cell preparations containing significant T cell contamination. This creates a problem because most in vitro work with NK cells requires the activation of these cells with a cytokine (IL-2) which also activates T cells (Naume and Espevik, 1994). Activated T lymphocytes have the same cytolytic effect as an activated NK cell in that they both lyse target cells. As a result it is very difficult to detect NK activity with a T cell contaminated NK cell preparation. A method of identifying these NK cells in a heterogenous population of cells needs to be developed. In our laboratory, a cytotoxicity method has been worked out by Nancy May (former graduate student), however this technique is not very specific for NK cells and it is very time consuming. An alternate method which can be pursued is a cell staining method which would allow labeling of specific cells. This technique involves labeling the cells with an antibody which is specific for a cell surface antigen. This antibody is then reacted with an anti-isotype (anti-antibody class; ie: anti-IgG) secondary

antibody coupled to an enzyme. This is then reacted with a chromogenic substrate which changes color at the site of the antibody for positive identification of the labeled cells. Several unsuccessful attempts with this method, using a commercially available primary antibody to asialoganglioside-GM1 (Asialo-GM1) have been made in our laboratory (see Results). The problems encountered may be attributed to the primary antibody which was a polyclonal antibody (serum from an immunized animal). Polyclonal antibodies generally contain all the antibody isotypes. In the literature there is some evidence suggesting that the IgM fraction may be more cross reactive with other gangliosides (Naiki et al., 1974). Therefore, nonspecific binding of the primary antibody may be the problem. To solve this problem we would like to develop a monoclonal antibody to asialo-GM1. Monoclonal antibodies are antibodies made by immunizing a mouse with whole antigen, then cloning each antibody producing cell so that each clone produces multiple copies of only one antibody with one unique antigen binding site. Due to the specificity of a monoclonal antibody, the cross reactivity seen with the polyclonal antibody may be eliminated or decreased. Monoclonal antibodies against the natural killer cell surface molecule asialoganglioside-GM1, are not commercially available; therefore, the only way to obtain this antibody was to make it in our laboratory.

Monoclonal Antibody against Asialoganglioside GM1

To make a hybridoma cell, spleen cells from an asialo-GM1 immunized mouse (containing B cells which are making antibodies to asialo-GM1) were

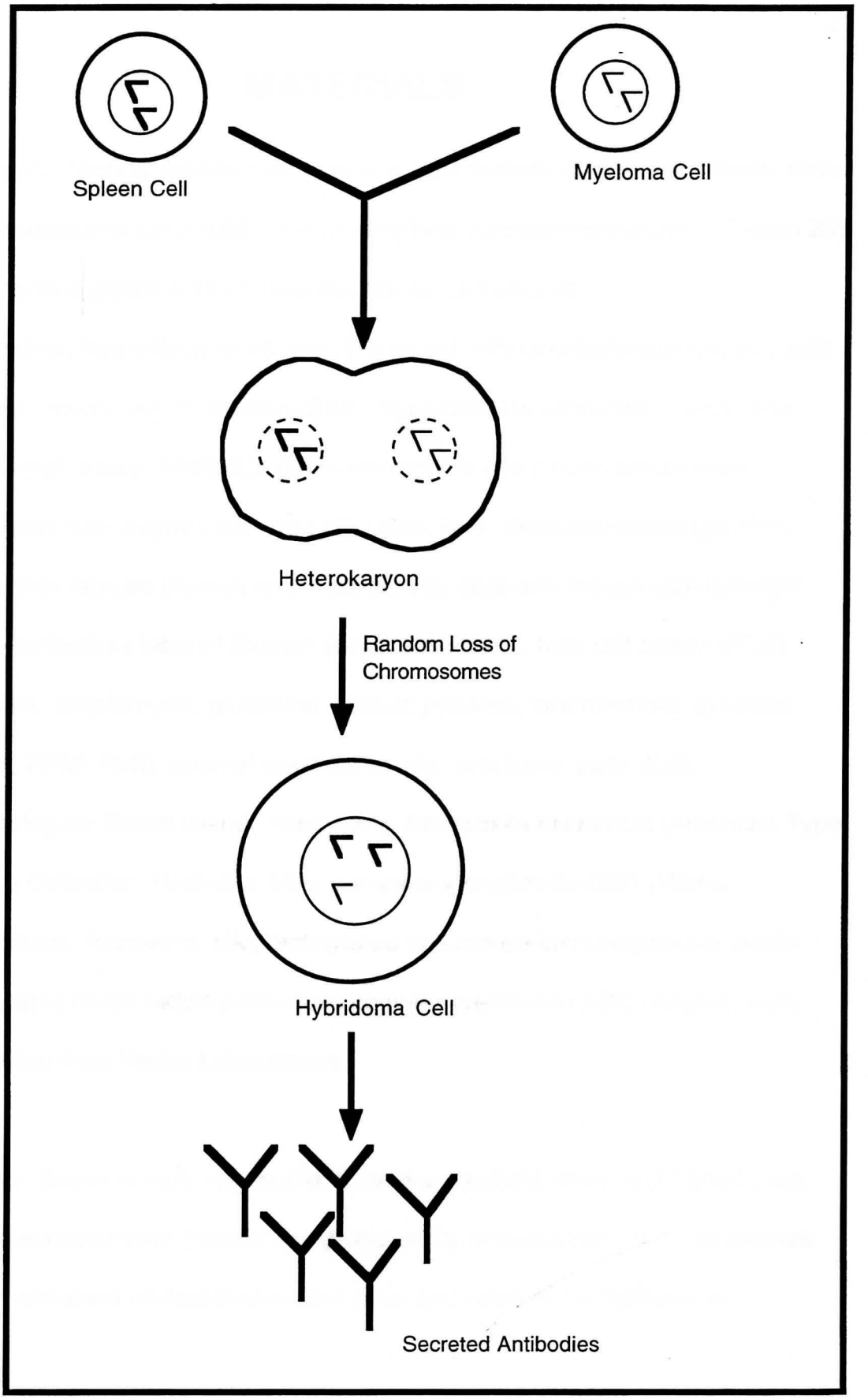
fused with a myeloma cell (cancer cell) (Figure 4). Myeloma cells are used in the fusion because they have the potential to proliferate for an indefinite period of time in culture. The resulting cell is called a heterokaryon, a multinucleated cell having two or more separate nuclei. In this state, the cells are very unstable because of the excess chromosomes. As the cell continues to divide it looses a variable number of chromosomes from each parent cell. Quite frequently, these cells will loose the chromosome which is vital for their survival or function; thus, loss of the clone of interest can occur frequently. The genes which are vital to our study are those that allow the cells to proliferate in culture, as well as those responsible for the secretion of the monoclonal antibody to asialo-GM1.

Summary

Natural killer cells are a subpopulation of cells which are able to lyse several types of tumor and virus infected cells without prior sensitization. As a result of their possible role in tumor surveillance, they have been the subject of numerous studies in mouse and human systems. Several years ago it was also discovered that NK cells express the cell surface marker molecule asialo-GM1. This molecule, which is expressed primarily on natural killer cells, is thought to play a role in the cytotoxic activity of natural killer cells. Currently, rabbit polyclonal antibodies are used to identify and remove NK cells in <u>in vivo</u> and <u>in vitro</u> studies. While polyclonal antibodies are a useful investigative tool, a more specific antibody can be obtained by utilizing hybridoma technology. In the present study we utilized hybridoma technology to produce a monoclonal

antibody which can be used as a research tool for further studies with natural killer cells.

Figure 4. Schematic overview of the formation of a hybridoma cell. This diagram depicts the fusion of a B cell from the spleen of an immunized mouse with a myeloma cell resulting in the formation of an hybrid cell.


Spleen Cell
Myeloma Cell
Heterokaryon
Random Loss of
Chromosomes
Hybridoma Cell
Secreted Antibodies

MATERIALS

Reagents: Sodium bicarbonate (tissue culture tested), sodium phosphate, citric acid, asialoganglioside-GM1, polyoxyethylene sorbitan monolaurate (Tween-20), polyethylene glycol, sodium chloride, ammonium chloride, Tris[hydroxymethyl]aminomethane (Tris-base), ethylenediaminetetraacetic acid (EDTA) , bovine serum albumin (BSA), hypoxanthine-aminopterin-thymidine (HAT), Histopaque-1080, 3,3' diaminobenzidine and mouse serum were purchased from Sigma Chemicals (St Louis, Mo). Goat-anti-rabbit IgG (H+L) peroxidase labeled (human serum absorbed), goat-anti-mouse IgG+IgA+IgM (H+L) peroxidase labeled (human serum absorbed), fetal calf serum (FCS), penicillin, streptomycin, glutamine, sodium pyruvate, oxaloacetate, pyruvate, Insulin, RPMI-1640, minimal essential media with Earls Salts (Life Technologies; Grand Island , New York); *Salmonella minnesota* (American Type Culture Collection, Rockville, MD); anti-asialoganglioside-GM1 (Wako Bioproducts, Richmond, VA); biotinylated anti-mouse immunoglobulin, avidin-biotinylated horse radish peroxidase complex (vectastain ABC reagent) were purchased from Vector Laboratories.

Animals: Seven to eight weeks old male New Zealand White and DBA/2 mice were purchased from Harlan Sprague-Dawley (Indianapolis, In). All animals were maintained on standard rodent chow and water in the facilities at

Youngstown State University.

Tumor Cells: P3X63-AG8.653 (myeloma, non-secreting mouse cells) were purchased from American Type Culture Collection (ATCC, Rockville, MD). These cells were maintained in RPMI-1640 media supplemented with 20% fetal calf serum, penicillin (100 U/ml), streptomycin (100 ug/ml) and glutamine (2 mM) in a 37°C, 5% carbon dioxide (CO_2) Incubator.

Feeder Cells: MRC-5 fibroblast cells (Harlow and Lane, 1988) were purchased from American Type Culture Collection (ATCC, Rockville, MD). These cells were maintained in minimal essential media (MEM) with Earls Salts supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 ug/ml), glutamine (2 mM) and sodium pyruvate (1 mM) at 37°C in a CO_2 incubator.

METHODS

A. Isolation of Lymphocytes for Use as Controls in Immunostaining

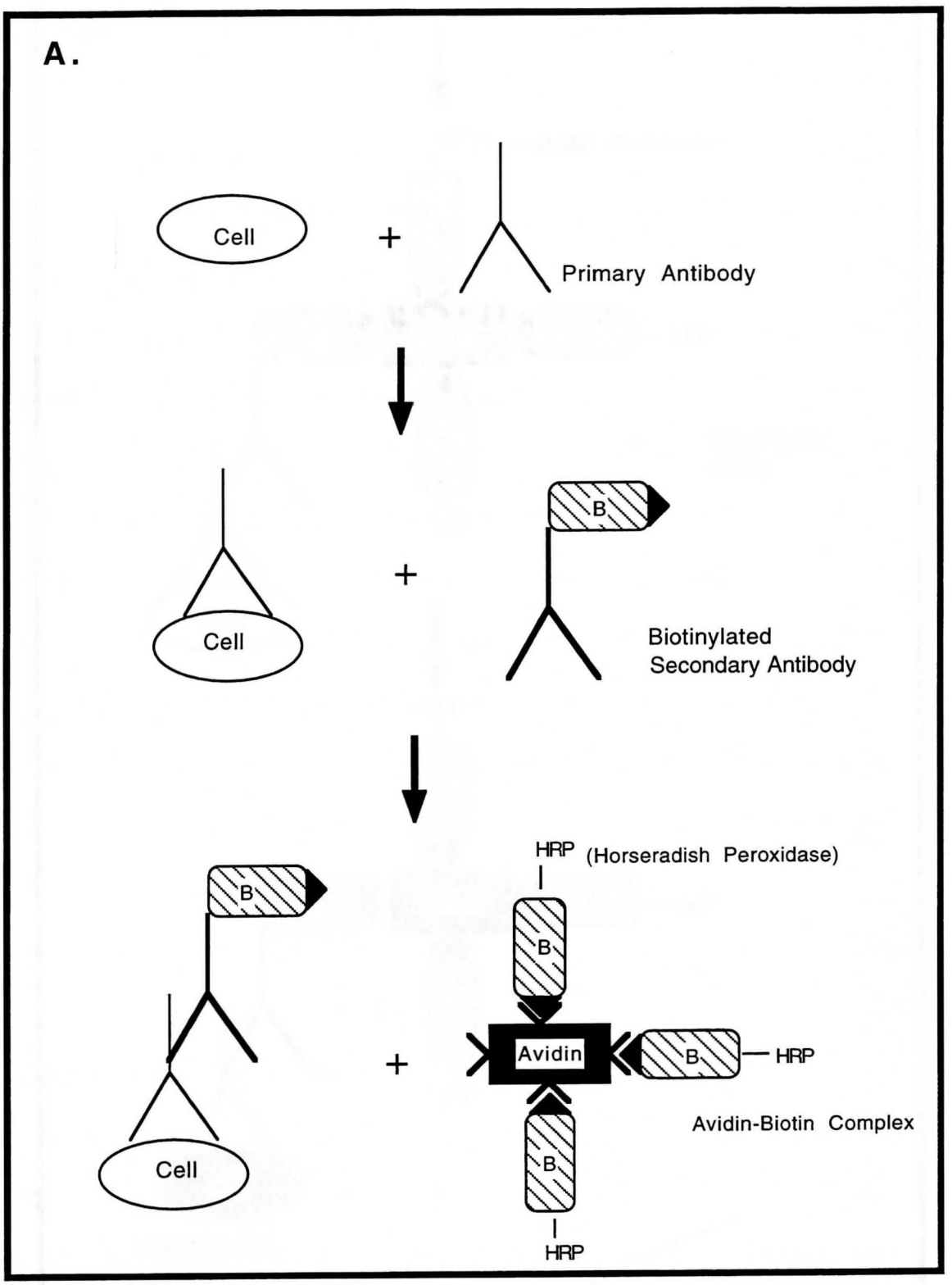
DBA/2 mice were euthanized using a carbon dioxide chamber. Blood was collected from the heart, the spleen was removed and placed in phosphate buffered saline (PBS: 0.1 M phosphate, 0.15 M sodium chloride) at physiological pH. Lymphocytes were isolated from blood by placing the (3 diluted) blood on a Histopaque (Ficoll) gradient. The spleen was washed and teased apart to release the cells into serum-free RPMI-1640 media and these cells were then placed on another histopaque gradient for mouse cells. Both ficoll gradients were centrifuged at 400 x g for 30 minutes to allow separation of lymphocytes from erythrocytes. The white buffy coat layer of each gradient (containing the lymphocytes) was collected and washed three times in PBS. After washing, the cells were resuspended in PBS containing 1% BSA (bovine serum albumin) at approximately 1 X 10^5 cells per 10 ul. A 10 ul aliquot of the resuspended cells were added to a clean slide and the cells were allowed to dry on the slides. The cells were fixed by incubating the slides in cold acetone for 10 minutes. The slides were stored at -20°C until needed.

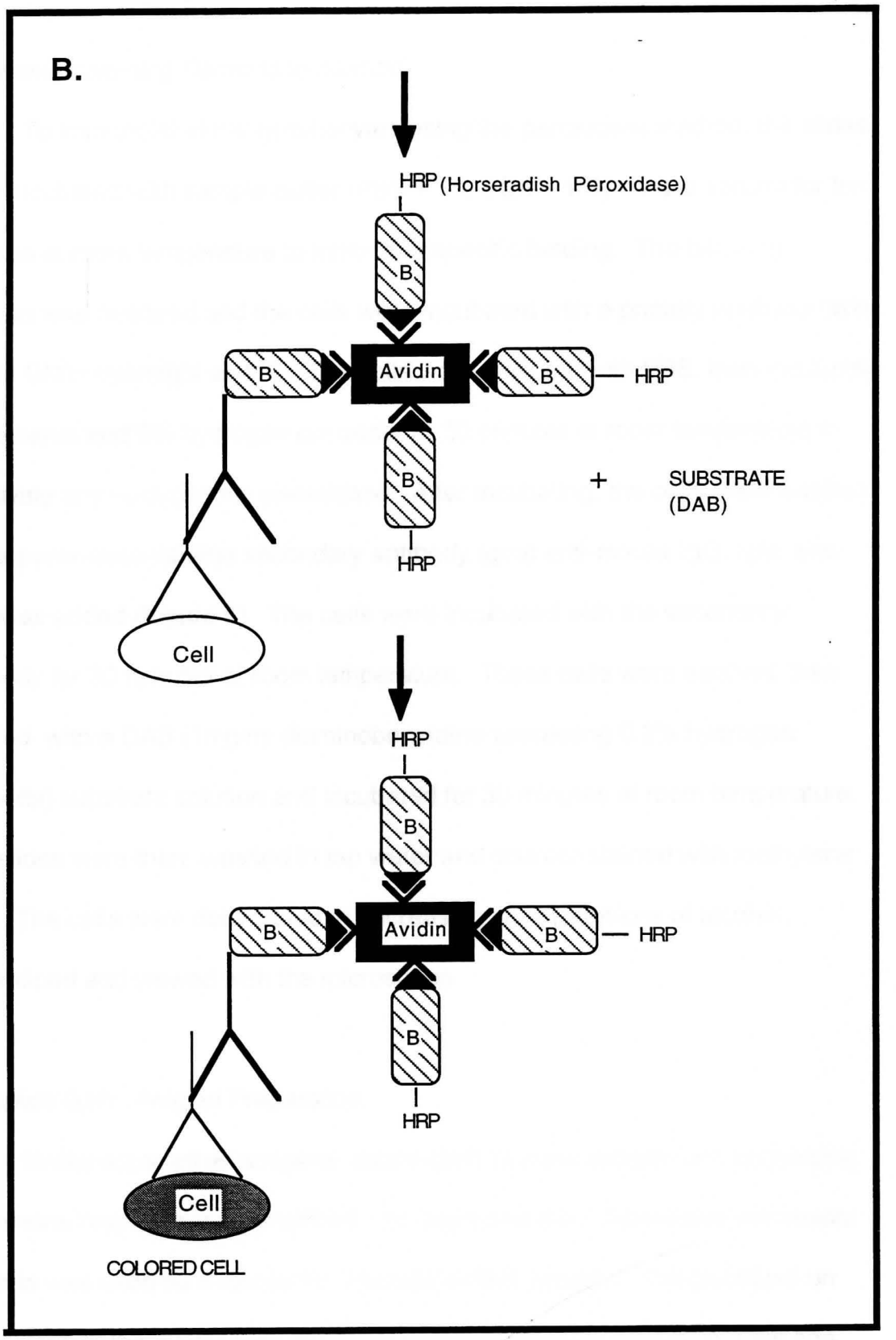
B. Immunostaining: Vectastain Method.

Cell samples on a slide were incubated with sample buffer (PBS + 1% BSA + 1:50 mouse serum) for ten minutes at room temperature to inhibit

nonspecific protein binding. The blocking solution was removed and the cells incubated overnight at 4°C with a primary antibody directed against natural killer cell asialo-GM1. The cells were washed with PBS, then incubated in methanol and 6% hydrogen peroxide for 30 minutes at room temperature to inactivate any endogenous peroxidase. After the incubation, the cells were washed and incubated with normal rabbit or goat serum for 30 minutes to prevent nonspecific binding of the secondary antibody (biotinylated anti-mouse immunoglobulin). Excess serum was blotted from the slide, then biotinylated secondary antibody was added and incubated for 30 minutes at room temperature (see Figure 5 A and B). The cells were washed and incubated for 30-60 minutes in Vectastain ABC reagent. The ABC reagent is avidin-biotinylated horseradish peroxidase complex which has been incubated at room temperature for 30 minutes. Avidin is a protein that has four binding sites for biotin. Prior to adding the ABC reagent, the avidin is mixed with biotin at a concentration that would theoretically leave only one avidin site available for binding to the biotin site on the secondary antibody. After allowing the avidin to bind to the secondary antibody, the cells were washed and incubated in a peroxidase substrate solution (1mg/ml 3, 3' diaminobenzidine containing 0.2% hydrogen peroxide) for 5-7 minutes at room temperature. The cells were washed in tap water and counter-stained with methylene blue. The cells were dehydrated in increasing concentrations of alcohol, coversliped and viewed with the microscope.

Figure 5 (A and B). Schematic of the steps in the Vectastain Reaction.


A.
Cell
+
Primary Antibody
Cell
+
B
Biotinylated
Secondary Antibody
B
Cell
+
HRP (Horseradish Peroxidase)
B
Avidin
B
HRP
B
HRP
Avidin-Biotin Complex


B.
HRP (Horseradish Peroxidase)
B
Avidin
B
HRP
B
HRP
+
SUBSTRATE
(DAB)
Cell
HRP
B
Avidin
B
HRP
B
HRP
Cell
COLORED CELL

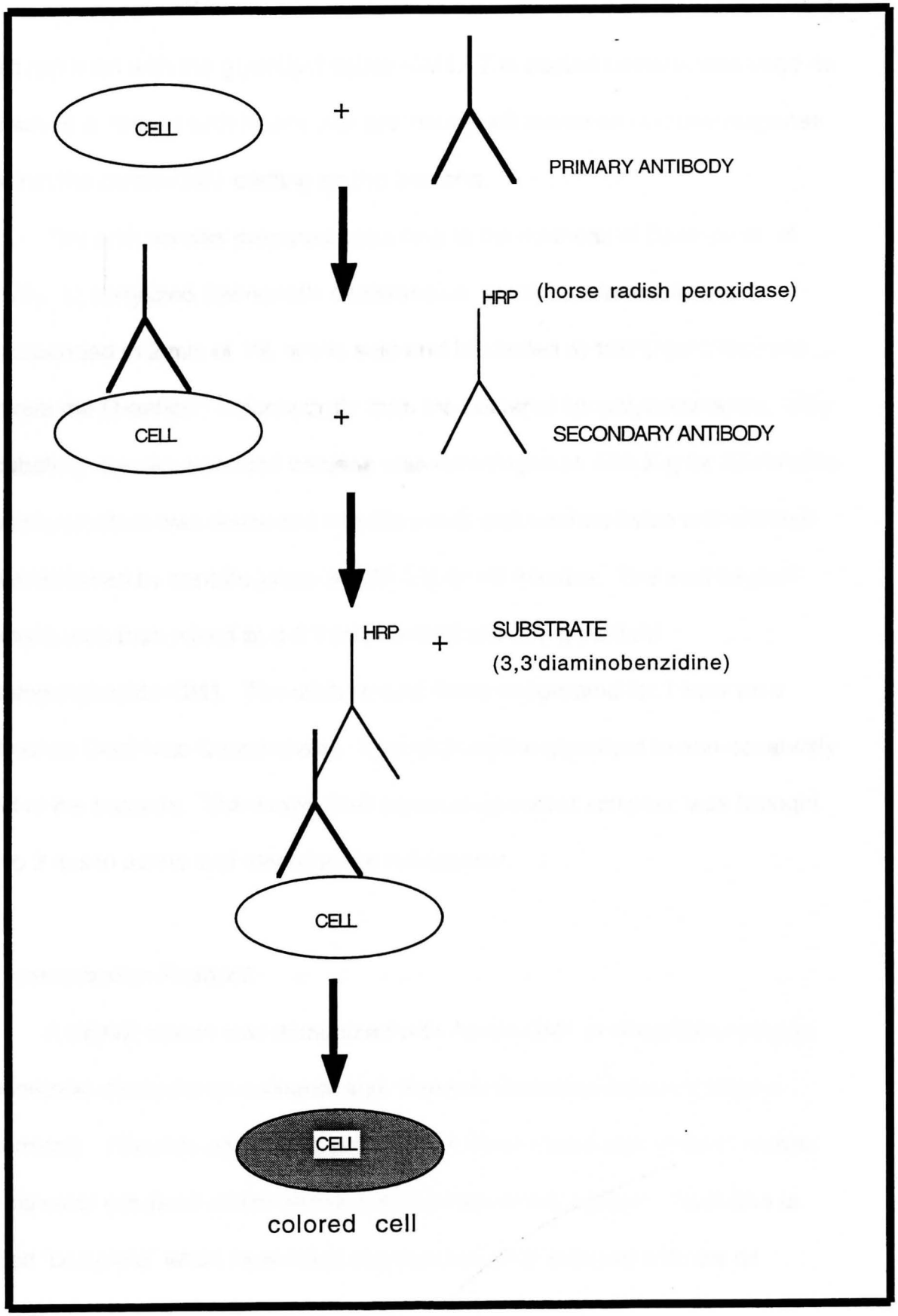
C. Immunostaining:Peroxidase method.

To immunolabel the lymphocytes using the peroxidase method, the slides were incubated with sample buffer (PBS + 1% BSA + 1:50 mouse serum) for ten minutes at room temperature to inhibit nonspecific binding. The blocking solution was removed and the cells were incubated with a primary antibody (anti-Asialo GM1) overnight at 4 °C. The cells were washed with PBS, then incubated in methanol and 6% hydrogen peroxide for 30 minutes at room temperature to inactivate any endogenous peroxidase. After incubating, the cells were washed and a peroxidase labeled secondary antibody (goat anti-mouse IgG, IgM, and IgA) was added (Figure 6). The cells were incubated with the secondary antibody for 30 minutes at room temperature. These cells were washed, then flooded with a DAB (1mg/ml diaminobenzidine containing 0.2% hydrogen peroxide) substrate solution and incubated for 30 minutes at room temperature. The slides were then washed in tap water and counter-stained with methylene blue. The cells were dehydrated in increasing concentrations of alcohol, coversliped and viewed with the microscope.

D. Asialo GM1: Antigen Preparation:

Unlike some other antigens, asialo-GM1 is a self antigen and stimulating an immune response is very difficult. To overcome this, *Salmonella minnesota* bacteria was used as a carrier for the asialo-GM1 antigen. The glycolipid on the surface of the *Salmonella minnesota* was removed by acid hydrolysis and

Figure 6. Schematic of the steps in the Peroxidase Reaction


CELL
+
PRIMARY ANTIBODY
HRP (horse radish peroxidase)
CELL
+
SECONDARY ANTIBODY
HRP
+
SUBSTRATE
(3,3'diaminobenzidine)
CELL
CELL
colored cell

then replaced with the glycolipid asialo-GM1. The coated bacteria was used to immunize a mouse with hopes that the mouse will mount an immune response against the asialo-GM1 coating on the bacteria.

The antigen was prepared according to the methods of Galanos et. al. (1979). Lyophylized *Salmonella choleraesius, serotype minnesota* was resuspended in 2 mls of 1% acetic acid and incubated at 100°C for 2 hours to remove the O-antigen carbohydrate from the bacterial lipopolysaccharide. After incubating, the resuspended bacteria was centrifuged at 570 X g for 10 minutes. The supernatant was discarded and the pellet was washed twice with distilled water followed by centrifugation at 570 X g for 10 minutes. The acid-treated bacteria was then mixed at a 4:1 ratio (wt/wt) with the glycolipid asialoganglioside-GM1. The mixture was rotary evaporated for 1 hour on a Labconco Centrivap Concentrator. This allowed the glycolipid to non-covalently bind to the bacteria. The evaporated bacteria-glycolipid complex was brought up to 3 mls in saline and stored in the refrigerator.

E. Immunization Protocol:

A DBA/2 mouse was immunized with Asialo-GM1 at 50ug/200ul (Sigma Chemicals) dissolved in methanol and Freunds Complete Adjuvant (Sigma Chemical). Freunds adjuvant is an oil which when mixed with antigen, makes an oil/water emulsion which allows slow release of the antigen. Freunds's is called "complete" when heat-killed mycobacterium is included with the oil

adjuvant to stimulate the immune system. One week later the animal was given a second intraperitoneal injection of asialo-GM1 with incomplete Freunds Adjuvant (50 ug/200 ul). Two days before fusion was to occur the mouse was injected intravenously with Asialo-GM1 (Hallow and Lane, 1988). A B cell hybridoma was created, as described below, and serum from the immunized animal and the supernatant of the B cell clones were screened for the production of antibodies to asialo-GM1 by the ELISA method. Unfortunately, neither the serum nor the supernatant of the clones contained antibody to asialo-GM1. Therefore, this experiment was aborted, and a new immunization protocol was tried before doing another fusion.

Seven to eight week old NZW (Shimada and Iwata, 1987) mice were immunized with asialoganglioside-GM1 non-covalently absorbed to naked *Salmonella minnesota*, as described above. Each mouse received the following intravenous injections of the glycolipid-bacteria complex in saline: 5 ug on day 0, 10 ug on day 4, 15 ug on day 7, and 20 ug on day 12. After a rest period of two weeks the mice received a final booster injection of 20 ug glycolipid-bacteria complex four days before the spleens were harvested (Young et. al., 1979). Each mouse was monitored throughout the immunization period for antibody production. Blood samples were collected and tested via enzyme-linked immunosorbent assay, as described below.

F. Hybridoma Protocol.

The procedure of somatic cell hybridization was employed to produce hybrid cells that can secrete monoclonal antibodies to asialoganglioside-GM1 (Hallow and Lane, 1988). Briefly, the immunized mouse was sacrificed and the spleen was removed aseptically into sterile, serum-free RPMI-1640 media. A single cell suspension was prepared by passing the spleen through a tissue sieve. The dispersed cells were allowed to sit until the large clumps settled out, then the supernatant was collected and centrifuged at 400 X g for 8 minutes. After centrifuging, the supernatant was discarded and the pellet was resuspended in 1 ml Tris-ammonium chloride (0.16M NH_4Cl, 0.17M Tris-base) to lyse any remaining red blood cells. This cellular suspension was layered over fetal calf serum and centrifuged at 300 X g for 10 minutes to remove the dead cells. After centrifuging, the cells on top of the fetal calf serum were collected and counted with the aid of a hemocytometer. The fusion partner, P3X cells in the growth phase (recently fed and rapidly dividing), were also collected and counted.

To make the hybrid cells, 1 X 10^8 spleen cells were combined with 8.76 X 10^6 hypoxanthine-aminopterin-thymidine (HAT) sensitive, non-secreting mouse P3X myeloma B cells (1:5) and centrifuged at 200 X g for 8 minutes. To fuse the cells, the supernatant was removed and 1 ml of a 50% (w/v) solution of polyethylene glycol (PEG) in RPMI-1640 was added to the cells over a period of one minute while shaking the tube. Serum-free, RPMI-1640 media (8 mls) was

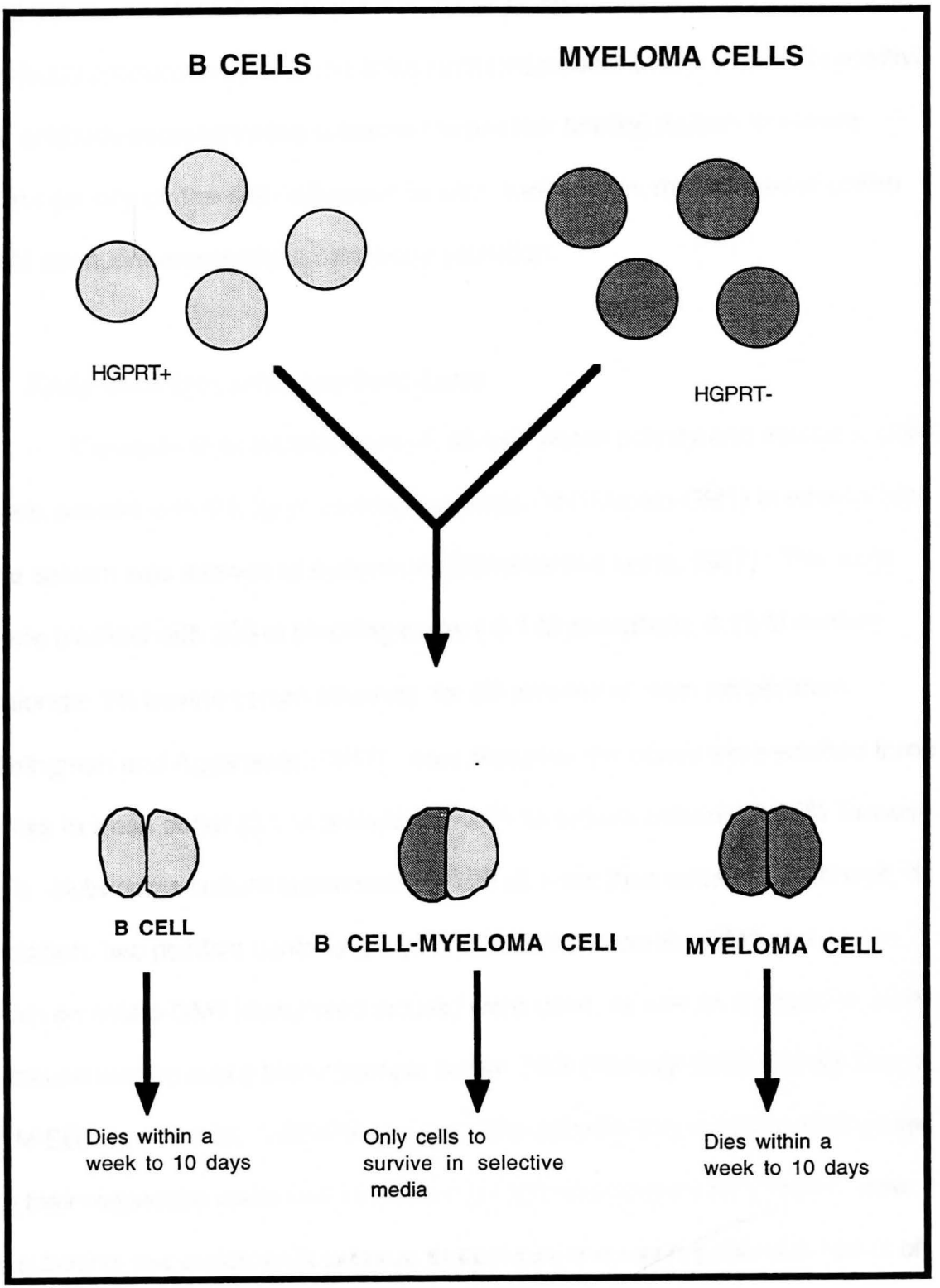
then added over a period of 5 minutes while shaking the tube. The cells were centrifuged at 200 X g for 8 minutes, then the pellet was resuspended in 24 mls of RPMI-1640 supplemented with 20% fetal calf serum, penicillin (100 U/ml), streptomycin (100 ug/ml) and glutamine (2mM). The fused cells were then seeded (1ml/well) into a 24 well, flat-bottomed falcon tissue culture plate which had a layer of MRC-5 feeder cells on the bottom. The feeder cells are adherent fibroblasts that provide nutrients and growth factors to the hybridoma cells (Morgan and Darling, 1993). The cells were incubated at 37°C overnight in 5% CO_2, 95% air Queue incubator.

The myeloma cell used is a B cell myeloma which lacks the ability to secrete antibodies and carries a selective marker. The selective marker is a defect in the enzyme hypoxanthine-guanidine phosphoribosyltransferase (HGPRT) which normally allows the cell to synthesize DNA via the salvage pathway. Mammalian cells have two pathways they can utilize to synthesize nucleotides: salvage and de novo. In the salvage pathway cells utilize the enzymes thymidine kinase and hypoxanthine-guanidine phosphoribosyltransferase to synthesize the necessary nucleotides from purine bases and thymidine. In the de novo pathway, cells synthesize purine and pyrimidine nucleotides from simpler carbon and nitrogen compounds rather than from already formed purine and pyrimidine. The early stages of this pathway involve the transfer of a methyl or formyl group from an activated form of tertrahydrofolate to a nucleotide precursor. This transfer step can be blocked

with the anti-folate drug aminopterin which forces the cell to use the salvage pathway. Therefore, when the $HGPRT^-$ myeloma cells are fused with the $HGPRT^+$ cells and grown in media containing hypoxanthine-aminopterin-thymidine (HAT), the aminopterin will block the de novo pathway which allows the cells to salvage the hypoxanthine and thymidine for DNA synthesis. Since only the spleen cells carry the genes for the necessary enzymes, only hybrid cells will survive in the HAT media. The unfused spleen cells will survive for only a short time in culture and all the $HGPRT^-$ myeloma cell will die (Figure 7).

HAT selection was performed by adding 1 ml of a 2 X HAT media [RPMI-1640 + 20% fetal calf serum + penicillin (100U/ml)/streptomycin(100ug/ml) + glutamine (2mM) + HAT (100uM hypoxanthine, 0.4uM aminopterin, 16uM thymidine) to the 1 ml of media in each of the 24 wells. After 5 days, the cells were fed 1X HAT media for about a week. Subsequent feedings were with RPMI-1640 supplemented with FCS, penicillin / streptomycin and glutamine until the cells were confluent. Once the cells were confluent, some of the media was removed and assayed for its antibody content using the enzyme-linked immunosorbent assay technique. Cells from the wells with clones secreting antibodies to asialoganglioside-GM1 were transferred to a fibroblast coated 96 well microtiter plate and serially diluted via limiting dilution (Hallow and Lane, 1988) such that only one cell should be present in several wells. These cells were allowed to grow and then screened for the number of colonies that developed per well. The cell were allowed to grow until they were confluent

Figure 7. Cell Fusion. This diagram illustrates the survival pattern of the various hybrids that result from a fusion in selective media.


B CELLS
MYELOMA CELLS
HGPRT+
HGPRT-
B CELL
B CELL-MYELOMA CELL
MYELOMA CELL
Dies within a week to 10 days
Only cells to survive in selective media
Dies within a week to 10 days

and the supernatant from the wells with five or less colonies were screened for antibody production by enzyme-linked immunosorbent assay. The wells positive for antibody secretion were subjected to another limiting dilution to assure homogeneity of the cell population in each well. Again, the cells were grown until confluent and tested for antibody secretion.

G. Enzyme-Linked Immunosorbent Assay:

The wells of a round-bottomed, 96 well falcon polystyrene microtiter plate were seeded with 0.5 ug of asialoganglioside-GM1(Asialo-GM1) in ethanol and the solvent was allowed to evaporate (Shimada and Iwata, 1987). The wells were blocked with 200 ul blocking buffer (0.1 M phosphate, 0.15 M sodium chloride, 1% bovine serum albumin) for 30 minutes at room temperature (Bringman and Aggarawal, 1987). After blocking, the plates were washed three times in wash buffer (0.1 M phosphate, 0.15 M sodium chloride, 0.05% Tween-20). Hybridoma culture supernatants (100 ul) were then added to each well. In addition, two positive controls (polyclonal rabbit anti-asialo -GM1 and serum from an asialo-GM1 immunized mouse) were used, as well as a negative control (mouse serum) and a blank [sample buffer: TBS (150 mM NaCl, 50 mM Tris, 2 mM EDTA), 1% BSA, 0.05%Tween-20]. The controls and samples were added to their respective wells and incubated at room temperature for 2 hours. After incubating, the plates were washed three times with wash buffer and 100 ul of peroxidase-conjugated anti-mouse (for hybridoma supernatant) or anti-rabbit (for

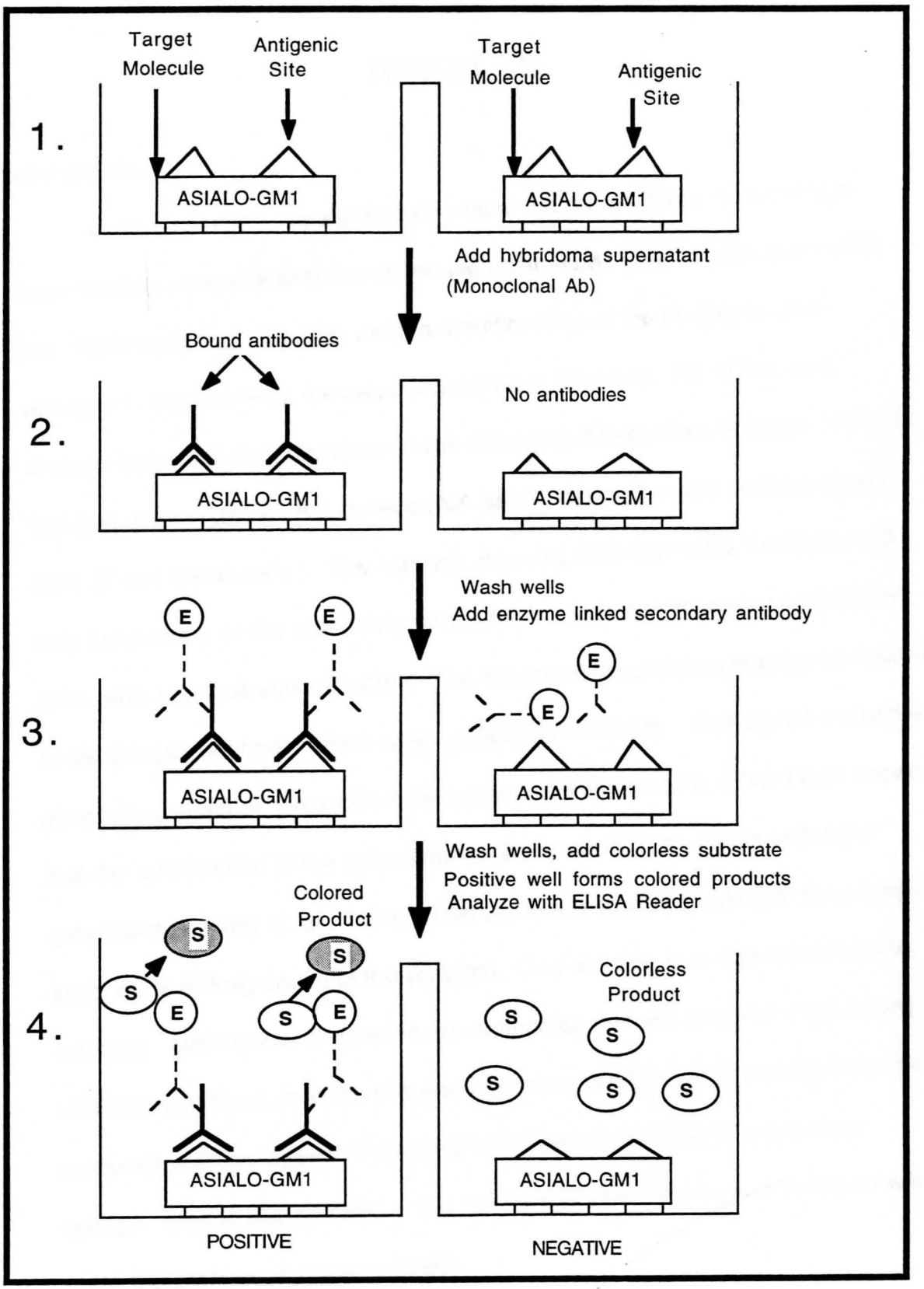
polyclonal asialo-GM1) immunoglobulin was added at a dilution of 1:1000 in sample buffer. The plates were incubated for 1 hour at room temperature, then washed three times with wash buffer. Next, 100 ul of o-phenylenediamine (0.1 mg/ml) in O.1 M phosphate/citrate buffer containing 0.02% hydrogen peroxide (H_2O_2) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by adding 50 ul of 2.5 N sulfuric acid and the absorbance read at 490 nm using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT) (see Figure 8).

H. Enzyme-Linked Immunosorbent Assay for Immunoglobulin:

The well of a round-bottom, 96 well falcon polystyrene microtiter plate were seeded with 100 ul of the supernatant from the various subclones. In addition, one well was seeded with mouse IgG (positive control) and another with buffer (blank) . The wells were blocked with 200 ul blocking buffer (0.1 M phosphate, 0.15 M sodium chloride, 1% bovine serum albumin) for 30 minutes at room temperature (Bringman and Aggarawal, 1987). After blocking, the plates were washed three times in wash buffer (0.1 M phosphate, 0.15 M sodium chloride, 0.05% Tween-20). To each well was added 100 ul of peroxidase-conjugated anti-mouse IgG, IgA and IgM (1:1000 dilution) in sample buffer. The plates were incubated for one hour at room temperature, then washed three times with wash buffer. Next, 100 ul of o-phenylenediamine (0.1 mg/ml) in 0.1 M phosphate/citrate buffer containing 0.02% hydrogen peroxide was added to each

well and incubated at room temperature for 30 minutes. The reaction was stopped by adding 50 ul of 2.5 N sulfuric acid and the absorbance read at 490 nm using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT).

Figure 8. Schematic overview of the basic steps in an Enzyme-Linked Immunosorbent Assay (ELISA). (1) Binding of antigen (Asialo-GM1) to the well; (2) Primary antibody binds to the antigen; (3) Enzyme-labeled secondary antibody binds to the primary antibody; (4) Substrate reacts with the enzyme to cause a color change.


1.
Target Molecule
Antigenic Site
ASIALO-GM1
Target Molecule
Antigenic Site
ASIALO-GM1
Add hybridoma supernatant
(Monoclonal Ab)
2.
Bound antibodies
ASIALO-GM1
No antibodies
ASIALO-GM1
Wash wells
Add enzyme linked secondary antibody
3.
E
E
ASIALO-GM1
E
E
ASIALO-GM1
Wash wells, add colorless substrate
Positive well forms colored products
Analyze with ELISA Reader
4.
Colored Product
S
S
E
S
S
E
ASIALO-GM1
Colorless Product
S
S
S
S
S
ASIALO-GM1
POSITIVE
NEGATIVE

RESULTS

Immunostaining

In these studies, we attempted to label the natural killer cells using a commercially available antibody to mouse asialo-GM1 (Wako Chemicals USA, Inc., Richmond, Va). Though various modifications of the protocols were attempted (see Table 2), the same results were obtained. All of the cells showed some staining, therefore it was difficult to differentiate between the cells that had asialo-GM1 on the surface (NK cells) versus the cells without asialo-GM1 (T cell and B cells). The controls included cells that were incubated with only the primary or the secondary antibody. In these control slides no labeled cells were seen, as was expected. The source of the problem may be attributed to the primary antibody, which was a polyclonal antibody. Polyclonal antibodies generally contain all the antibody isotypes. In the literature, it has been shown that the IgM fraction of the polyclonal antibody often cross reacts with other gangliosides (Naiki et. al., 1974). Therefore, nonspecific binding of the primary antibody is a likely cause of the problem. One solution is to use a monoclonal antibody. Monoclonal antibodies are antibodies that are made by immunizing with whole antigen, then cloning each antibody producing cell so that each clone produces multiple copies of only one antibody which binds to one unique epitope. Due to this specificity, it is hoped that using this type of antibody will solve the problem of cross reactivity.

Table 2
Immunostaining Data

Experiment #	Method	1° Antibody	Cells	Modifications	Results
1	Vectastain	∝ -AsialoGM1	Spleen Peripheral blood	------	All cells labeled *Control and **Experimental
2	Vectastain	∝ -AsialoGM1	Spleen Peripheral blood	Add mouse serum to sample buffer	Control unlabeled; Experimentals: all cells labeled
3	Vectastain	∝ -AsialoGM1	Spleen	1° Antibody in sample buffer	Control unlabeled; Experimentals: all cells labeled
4	Vectastain	∝ -CD3	Spleen	Labels anti-T cell surface molecule	Controls and Experimental all stained
5	Vectastain	∝ -AsialoGM1 ∝ -CD3	Spleen Peripheral blood	no counterstaining	Controls slightly stained; Experimental all stained
6	Peroxidase	∝ -AsialoGM1	Spleen	New secondary antibody	Controls unlabeled; Experimental all labeled
7	Vectastain	∝ -AsialoGM1 ∝ -CD3	Spleen	Used vectastain blocking reagent	Controls unlabeled; Experimentals all labeled

Note all cells were counterstained with Wright-Giemsa stain with the exception of experiments 5-7.
* Controls were cells exposed to only primary antibody or secondary antibody
**Experimentals were cells exposed to both primary and secondary antibody (Ab).

Anti-Asialo-GM1 Hybridoma Cells

The fusion of P3X63.Ag68.653 myeloma cells with spleen cells from a NZW mouse immunized with asialo-GM1, yielded twenty-four wells of cells. Once the cells had grown to confluency, the supernatants were tested for antibody against asialo-GM1. The ELISA results for these clones can be found in Table 3. Fifteen of the twenty-four wells tested were positive for antibody against asialo-GM1 (Figure 9), and the four with the strongest antibody reaction (A4, C2, D5, and D6) were subcloned via limiting dilution. This involved taking a 100 ul aliquot of cells from each positive well and serially diluting it 1:2 down and across a 96-well culture dish. The remaining cells were grown up in large batches and a small portion was processed for storage in the -70°C freezer. The subcloned cells were grown until confluent and then analyzed via the ELISA test for antibody secretion against asialo-GM1. Only the wells with 1-5 colonies were tested. In our first subcloning experiment, none of the wells were positive for anti-asialo-GM1 antibody. We believe that at some point during the culturing process the positive clones died or there were not any antibody secreting cells in the wells we selected for testing. Therefore, stocks of the pooled hybridoma cells which were positive in the first ELISA test were selected to be cultured and were re-subcloned.

Fourteen of the re-cultured cells (Table 4) were re-analyzed by ELISA for the production of antibodies against asialo-GM1. Of the fourteen

Table 3
Pooled Hybridoma Cells, 1/16/96
ELISA results for antibody
directed against Asialo-GM1

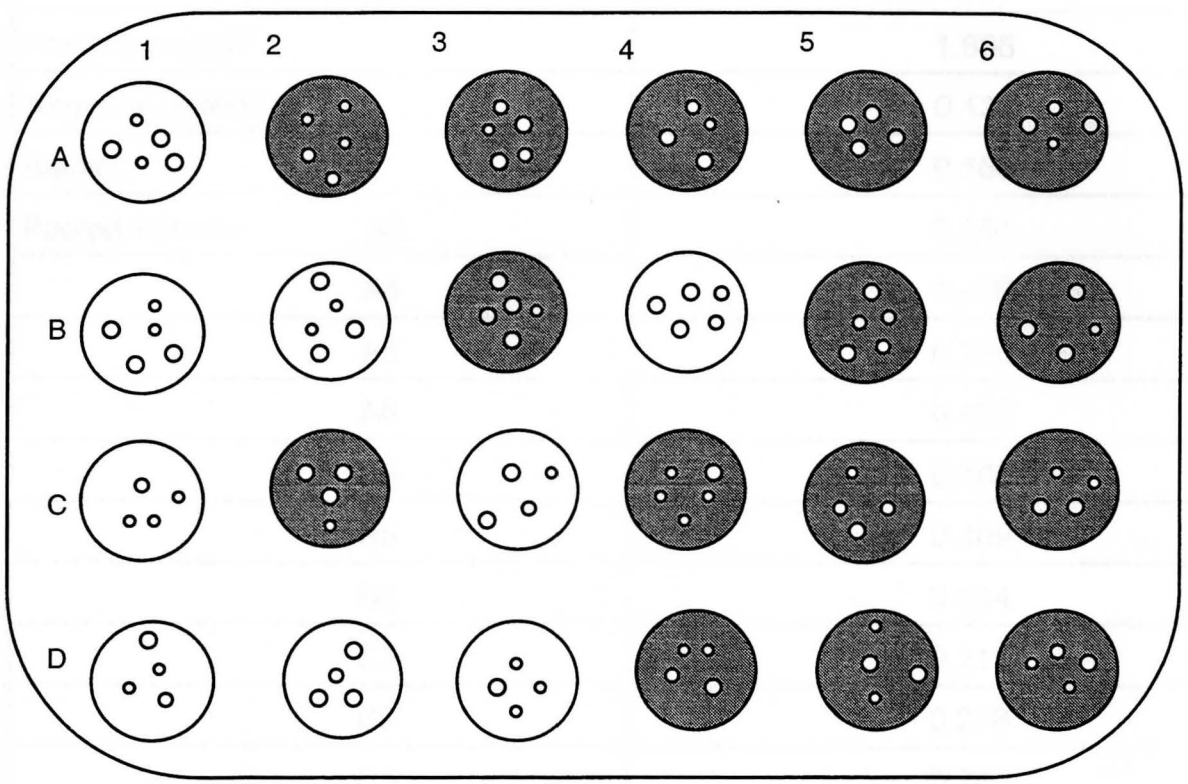
Samples	OD_{490} - Blank
Serum[a]	1.400
Positive control[b]	*******
Negative control[c]	0.757
Blank[d]	-------
Pooled Hybrids: A1	0.555
A2	1.615
A3	0.867
A4	1.893
A5	1.319
A6	1.254
B1	0.728
B2	0.627
B3	0.895
B4	0.742
B5	1.131
B6	1.222

Table 3 continued.

Samples	OD_{490} - Blank
C1	0.783
C2	2.565
C3	0.719
C4	1.224
C5	1.208
C6	1.128
D1	0.626
D2	0.609
D3	0.615
D4	0.964
D5	******
D6	2.163

[a] Serum from a mouse immunized with asialo-GM1
[b] Commercial polyclonal antibody to asialo-GM1
[c] Mouse serum
[d] Sample buffer (see 'Materials and Methods').
[e] ELISA reading which is outside the parameter of the ELISA Reader (too high).

Figure 9. Schematic diagram of the 24-well culture dish. Shaded circles represent wells containing cells that were determined by ELISA to be secreting ∝-Asialo-GM1 antibodies.



= Positive for monoclonal Ab to AsGM1

Table 4.
Pooled Hybridoma Cells, 3/8/96
ELISA results for antibody
directed against Asialo-GM1

Sample		OD_{490} - Blank
Anti-AsGM1 serum[a]		0.277
Positive control[b]		1.886
Negative control[c]		0.138
Blank[d]		0.166
Pooled Hybrids	A2	0.581
	A4	0.406
	A5	0.393
	A6	0.492
	B3	0.386
	B5	0.409
	B6	0.204
	C2	0.215
	C4	0.278
	C5	0.740
	C6	0.197
	D4	0.433
	D5	0.412
	D6	0.295

The ELISA was conducted by coating the wells of a tissue culture dish with asialo-GM1 and adding the supernatant of hybrid cells according to the procedure in 'Materials and Methods'.
[a]Serum from mouse immunized with asialo-GM1.
[b]Commercial antibody to asialo-GM1.
[c]Mouse serum
[d]Sample buffer (see 'Materials and Methods')

wells tested, five positive for the production of anti-asialo-GM1 antibodies were pursued for subcloning (Table 5). All wells with an ELISA reading with was less than three times the blank were considered negative. These were a heterogenous population of pooled hybridoma cells, therefore, further culturing was done to get a more homogenous population of cells. All the clones which were positive for anti-asialo-GM1 antibodies were subcloned via limiting dilution (Appendix A-E). These cells were subcloned in round bottom culture dishes which made it difficult to determine which wells had only one colony of cells. Only wells with 1-5 colonies were selected for testing via the ELISA test. Of the wells selected for testing, only a few were positive for anti-asialo-GM1 antibodies (Table 6). The subclones in Table 7 are the ones which are positive for the secretion of antibody against asialo-GM1. From this group of clones hybrid A2 (HA2) had only one clone which was positive and that was B12. HA6 and HD4 did not have any positive clones and HB5 had only one positive clone (F7). HC5 had eight positive clones (A6, A7, B11, B12, C11, D8, D9, H9). The number of positive clones were low; however, this is expected since as the cell divides it may loose chromosomes which are important in antibody secretion.

Table 5.
Pooled Hybridoma Cells, 3/8/96
ELISA positive for antibody
directed against Asialo-GM1

Pooled Hybridoma Cells	**OD_{490} - Blank**
A2	0.581
A6	0.492
B5	0.409
C5	0.740
D4	0.599

(See Table X for the blank, anti-AsGM1 serum, and positive and negative control OD).

Table 6
ELISA of the First Subclones for
Antibody directed against Asialo-GM1

Sample	OD_{490} - Blank
∝-Asialo GM1	0.296
Positive Control	0.947
Blank	------
Subclones HC5-A6	0.296
A7	0.266
B11	0.281
C11	0.286
D8	0.295
D9	0.293
H9	0.277
Subclone HA2-B12	0.308
∝-Asialo GM1	0.421
Positive Control	1.023
Blank	------
Subclone HB5-F7	0.142

Negatives for subclone HC5 were OD values less than 0.110 and 0.007 for HA2.

Table 7
First Subclones of the Pooled Hybridoma Cells
Clones Positive for ∝-ASGM1 Antibody

	Hybridoma Cells Positive First Subclones				
	HA2	HA6	HB5	HC5	HD4
Subclones	B12	----	F7	A6	----
				A7	
				B11	----
	----	----	----	B12	----
	----	----	----	C11	----
	----	----	----	D8	----
	----	----	----	D9	----
	----	----	----	H9	----

Subclones of the various pooled hybridoma cells which were positive for antibody secretion to asialo-GM1. The blank is 0.113 (Sample buffer)

One problem encountered in the analysis of the ELISA results was finding a good negative control. Early in our studies the positive clones were determined to be any cells with an ELISA result which was three times the blank however, this was not the most appropriate way of selecting the positive clones. Several unsuccessful attempts were made with mouse serum and mouse immunoglobulin IgG. It was later determined that the mouse serum was contaminated thus accounting for the positive ELISA response. To make a more accurate determination of which clones were actually negative for antibody to asialo-GM1, supernatants for the ELISA test were also tested for immunoglobulins which were not directed against asialo-GM1. Those clones which tested positive for immunoglobulin but not against asialo-GM1 were considered to be the negative controls for the respective clones. Positive clones were those with an ELISA result that was three times the blank.

All of the subclones which tested positive for antibody to asialo-GM1 (Table 7), were taken through a second subcloning process (Appendix F-J). Only the wells with one colony were further analyzed via the ELISA test for the secretion of antibody to asialo-GM1. Subclone HC5-A6 (derived from pooled hybrid C5) did not yield any wells with single colonies and will have to be subcloned again. HC5-A7 and H9 were subcloned a second time; however, they still need to be tested for antibody production by the ELISA test. Subclones HA2-B12, HB5-F7, and HC5- B11, B12, C11, D8, and D9 (all derived from pooled hybrid A2) were all tested by the ELISA method for antibody production to

asialo-GM1. Using the criteria described above for positive and negative clones, the following results were obtained after the second subcloning process.

The subclones of HA2-B12 which was derived from the pooled hybrid A2 and subclone B12 did not yield any clones which were positive for antibody against asialo-GM1 (Table 8). However, supernatant from the subclones F1, and H7 showed significant non-asialo GM1 immunoglobulin secretion (Table 9).

The HC5-B11 subclones which were derived from the pooled hybrid C5 and subclone B11, yielded only one positive clone which was G9 (Table 10). The ELISA result from clone G9 was 0.419, or 37% of the positive control. This is 5% higher than the ELISA result for the anti-ASGM1 serum which contains polyclonal antibody. Of these subclones only G10 show significant immunoglobulin secretion which was not specific for asialo-GM1 and it was considered to be the negative control for this hybrid HC5-B11 (Table 11).

Table 8.
Subclones of HA2-B12
ELISA result for antibody
directed against Asialo-GM1

Sample	OD_{490} - Blank	% of Positive Control
Anti-AsGM1 Serum	0.358	32
Positive control	1.117	100
Blank	* 0.113	----
B12	0.109	10
D11	0.068	6
D12	0.105	9
E12	0.206	18
F10	0.202	18
G8	0.201	18
H5	0.127	11
H7	0.256	23
H8	0.177	16

These are the second subclones of the clone HA2-B12. Based on the ELISA result none of these clones are positive for antibody against asialo-GM1.
*Blank

Table 9.
Subclones of HA2-B12
Result of ELISA for Immunoglobulin

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.132	------
Positive control	1.004	100
B12	0.458	45
D11	0.353	35
D12	0.306	30
E12	0.634	63
F10	0.727	72
G8	0.674	67
H5	0.300	29
H7	0.694	69
H8	0.448	44

These subclones were tested for immunoglobulin seretion which was not against asialo-GM1. Tthe secondary antibody used was anti-mouse IgG, IgA, and IgM. F10 and H7 have significant immunoglobulin response however, it's inconclusive because none of the corresponding clones had an ELISA response.

Table 10.
Subclone of HC5-B11
ELISA results for antibody
directed against Asialo-GM1

Sample	OD_{490} - Blank	% of Positive Control
Anti-AsGM1 serum	0.358	32
Positive control	1.117	100
Blank	0.113	----
F10	0.184	16
F11	0.151	13
F12	0.235	21
G9	0.419	37
G10	0.088	7.8
G11	0.284	25
G12	0.187	16

Subclone HC5-B11 yielded one positive clone: G9. It is 37% of the positive control.

Table 11
Subclones of HC5-B11
Results of ELISA for Immunoglobulin

Samples	OD_{490} - Blank	% of Positive Control
Blank	0.132	------
Positive control[a]	1.004	100
F10	0.590	58
F11	0.611	60
F12	0.720	71
G9	0.678	67
G10	0.714	71
G11	0.781	77
G12	0.658	65

[a] **Positive Controls are well coated with commercially available mouse IgG and reacted with anti-IgG, IgM and IgA. These are the immunoglobulin results for the subclones of HC5-B11 and based on the data only G10 would be a good negative control for this clone.**

Subclones of HC5-C11 which were derived from the pooled hybrid cells C5 and subclone C11 were determined to have two positive clones: G12 and H7 (Table 12). G12 had an ELISA reading of 0.316 which is 27% of the positive control and H7 was 0.316, 28% of the positive control. Table 13 shows the immunoglobulin results for the subclones of HC5-C11 with H8 being the only clone which had significant non-asialo GM1 immunoglobulin response. Therefore, H8 would be the negative control for HC5-C11.

HC5-D8 did not yield any positive clones (Table 14 and 15), however the immunoglobulin test of subclones B12, E12, F11 and G10 yielded significant non-asialo GM1 results. The subclones of HC5-D9 yielded two positive clones: D12 and F9 (Table 16). The ELISA result of each, respectively, were 0.348 (31% of positive control) and 0.329 (29% of the positive control). The negative control for this subclone was E12 as is evident in Table 17. E12 is the only clone with significant immunoglobulin response which is not directed against asialo-GM1. The last of the hybrids tested was HB5-F7 and it did not have any clones secreting antibodies to asialo-GM1.

The data presented demonstrate that from the pooled hybrid cells we created, five clones were isolated (Table 18) which have been determined by ELISA to be secreting antibodies to the NK cell surface molecule asialo-GM1. The isotype and specificity of these monoclonal antibodies are yet to be determined

Table 12.
Subclones of HC5-C11
ELISA results for antibody
directed against Asialo-GM1

Sample	OD_{490} - Blank	% of Positive Control
Anti-AsGM1 serum	0.358	32
Positive control	1.117	100
Blank	0.113	----
E10	0.109	9
F10	0.154	13
F11	0.135	12
F12	0.127	11
G7	0.266	24
G9	0.151	13
G12	0.310	27
H6	0.272	24
H7	0.316	28
H8	0.111	10

Subclone G12, and H7 were positive for antibody against asialo-GM1. H8 which did not have any antibody against asialo-GM1 had significant immunoglobulin to be used as a negative control for this clone.

Table 13.
Subclones of HC5-C11
Results of ELISA for Immunoglobulin

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.132	------
Positive control	1.004	100
E10	0.321	32
F10	0.674	67
F11	0.675	67
F12	0.641	64
G7	0.760	76
G9	0.631	63
G12	0.819	81
H6	0.819	81
H7	0.728	72
H8	0.783	78

These are the immunoglobulin result of the subclones of HC5-C11. Only H8 would be a good negative for this clone.

Table 14.
Subclones of HC5-D8
ELISA result for antibody
directed against Asialo-GM1

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.113	------
Positive control	1.117	100
Anti-AsGM1 serum	0.358	32
B12	0.260	23
D12	0.179	16
E11	0.132	11
E12	0.162	14
F11	0.185	16
F1 2	0.140	12
G10	0.219	20

These subclones did not yield any anti-asialo-GM1 positive cells, therefore they were disgarded.

Table 15.
Subclones of HC5-D8
Result of ELISA for Immunoglobulin

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.123	------
Positive control	1.004	100
B12	0.791	79
D12	0.643	64
E11	0.389	38
E12	0.717	71
F11	0.730	73
F12	0.572	57
G10	0.705	70

Since HC5-D8 did not yield any positive subclones, determination of a good negative control for this clone was not possible.

Table 16.
Subclones of
HC5-D9 and HB5-F7
ELISA result for antibody
directed against Asialo-GM1

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.113	------
Positive control	1.117	100
Anti-AsGM1 serum	0.358	32
HC5-D9		
D12	0.348	31
E12	0.157	14
F9	0.329	29
F10	0.158	14
G7	0.186	16
G9	0.184	16
H6	0.157	14
H7	0.190	17
H8	0.190	17
HB5-F7/H8	0.123	11

HC5-D9 yielded two positive clones: D12 and F9 which respectively was 31 and 29 percent of the positive control. HB5-F8 did not have any positive clones.

Table 17.
Subclones of
HC5-D9 and HB5-F7
Results of ELISA for Immunoglobulin

Sample	OD_{490} - Blank	% of Positive Control
Blank	0.132	------
Positive control	1.004	100
HC5-D9		
D12	0.678	67
E12	0.762	76
F9	0.801	79
F10	0.713	71
G7	0.365	36
G9	0.632	63
H6	0.703	70
H7	0.712	71
H8	0.666	66
HB5-F7		
H8	0.284	28

Based on the immunoglobulin and ELISA results it was determined that subclone E12 was the better negative control for the clone HC5-D9. The result on HB5-F7 was inconclusive.

Table 18
Second Subclones
ELISA positive for antibody
directed against Asialo-GM1

Subclones	Positive Second Subclones
HA2-B12	-------
HC5-B11	G9
HC5-C11	G12, H7
HC5-D8	-------
HC5-D9	D12, F9

These are the five subclones which were determine to be secreting antibody against asialo-GM1.

DISCUSSION

In this study we used spleen cells of the asialo-GM1 primed mouse, to create a hybrid cell which has the ability to secrete antibodies against asialo-GM1. Asialo-GM1, a NK cell surface molecule which has been identified on murine natural killer cells (Kasai et.al., 1980), was used to immunize the mouse prior to the fusion. As an antigen, asialo-GM1 is not very immunogenic for the following reasons. First, asialo-GM1 is a glycolipid: a class of molecule which is composed of a short chained carbohydrate covalently linked to a lipid, frequently found in the plasma membrane. In addition, asialo-GM1 is a self antigen and thus it is difficult to induce an autoimmune response to this particular antigen. To bypass these problems, the asialo-GM1 glycolipid was used to replace the glycolipid of a naked (no carbohydrate found on lipopolysaccharide)*Salmonella minnesota* bacteria. This asialo-GM1-bound *Salmonella minnesota* was used as an antigen with the hopes that the bacteria would increase the immunogenicity of the asialo-GM1. Spleen cells from a mouse, repeatedly immunized with asialo-GM1 were fused with myeloma B cells which was HGPRT- and Ab- to create a hybridoma cell line. The antibodies that a hybridoma cell clone secretes are monoclonal antibodies (mAb) which are generally directed against a specific site on the antigen (asialo-GM1).

In our studies we have isolated five subclones from the pooled

hybridoma cells which were determined by the ELISA test to be secreting antibody to asialo-GM1. In order to use the products (mAb) of these subclones as an investigative tool for further studies of natural killer cells, the monoclonal antibody will have to be characterized and purified. As with other hybridoma studies cited in the literature (Solomon and Higgins, 1987), bulk quantities of this antibody need to be prepared. Large amounts of the mAb can be produced by either bulk tissue culture or by production of ascites. For bulk culturing, the hybridoma cells are progressively transferred to larger containers which results in the production of large quantities of supernatants which contain the secreted antibody. The concentration of antibody obtained with this method is fairly low (10 - 15 ug/ml). The alternative method is to grow the hybridoma cell in the peritoneal cavity of an animal of the same strain as the tumor cell line and the spleen cell donor, resulting in the secretion of mAb within the abdominal cavity of the animal. To assure that this process will occur, the animals are primed with a substance (pristane or incomplete Freund's Adjuvant) which activates the peritoneal macrophages causing them to produce cytokines which assist in the growth of the hybridoma cells. This method tends to yield approximately 10 mg/ml of mAb, however the amount of contaminants obtained with this method is much higher.

Monoclonal antibodies can be purified by various methods, two of which will be discussed briefly. One method used for the purification of monoclonal antibodies is salt fractionation. Salt fractionation techniques relie upon the fact

that salt ions disrupt the hydrophilic bonds that form between proteins and water. Thus, as the salt-water reaction becomes more stable, the protein precipitates out of solution. Large molecules precipitate at lower salt concentration, so immunoglobulins (150 KD or more) can be precipitated from serum samples or culture supernatants, leaving the major contaminants in solution. Once the precipitant has been recovered, it can be dialyzed to remove any salts, giving a purified concentrated immunoglobulin. Another method used in the purification of mAb is affinity chromatography. In affinity chromatography, a specific protein binding molecule is attached to an agarose bead and is used to make a column for isolating the specific mAb. The protein usually used to purify antibodies is staphylococcus protein A (Staph protein A). Staph protein A binds to the Fc portion of an antibody. When serum or culture supernatant is added to the column, the Staph protein A binds to the Fc (functional) portion of the antibody allowing other proteins to be washed through the column. The antibody can then be eluted from the Staph protein A column by changing the pH of the buffer. Once the mAb has been purified, further characterization by isotype and specificity must be done. Isotype characterization involves determining the specific immunoglobulin (Ig) class (IgG, IgM. IgA, IgE etc.) of the monclonal antibody. This can be accomplished by utilizing a modified ELISA test where the wells of an ELISA plate are coated with various anti-immunoglobulin antibodies (ie anti-IgG, anti-IgM etc.) and the mAb's (supernatant of the clones) are reacted with them to determine their

immunoglobulin class (isotype). The immunoglobulin class which is seen with a mAb from a hybridoma cell is dependent to some extent on the frequency and timing of the immunization as well as the immunogenicity of the antigen. Immunization once or twice usually elicits a primary response, resulting in secretion of an IgM mAb. Three or more immunizations will stimulate a secondary response resulting in the secretion of an IgG mAb. Based on the immunization method implemented in this study I usually would expect that the predominant immunoglobulin class for our mAb should be IgG. However, the antigen is a lipid, which doesn't prime T cells and probably will only stimulate an IgM response.

As stated earlier, asialo-GM1 is a glycolipid which is composed of a carbohydrate and a lipid moiety. A monoclonal antibody can be against any portion of this molecule. Based on the carbohydrate or lipid composition of a glycolipid, there are various types of glycolipids within a mammalian system. Within the same family, they all share the same general composition and on occasion they have conserved regions which can cause a mAb against one glycolipid to cross react with another. The glycolipid used in this study is a ganglioside. Gangliosides are a family of glycosphingolipids that are composed of a sphingolipid (ceramide with a carbohydrate chain) substituted with one or more sialic acids. Classification of the gangliosides is determined by the number of sialic acid residues and their distribution on the molecule. The molecule asialo-GM1, which is also known by the chemical name of ganglio-N-

very similar to ganglioside GM1 which has one substituted sialic acid residue. Two other molecules with similar backbones have also been indentified: GD (disubstituted ganglioside) which has two sialic acid residues and GT (trisubstituted ganglioside) which has three sialic acid residues. Within the di- and tri-substituted molecules there exist mono- (one or more sialic acid residues on two different galactose molecules) and di-substituted (two sialic acid residues on the same galactose molecule) galactose units. A GD molecule which has monosubstituted galactose units is termed GD1a and the GD with disubstituted units is GD1b. In addition, there is GM2 and asialo-GM2 (ganglio-N-triosylceramide) as well as GM3 and asialo-GM3 molecules which differ from GM1 by the loss of one (or more) galactose residues. All of these gangliosides share the same general carbohydrate and lipid moiety and therefore, some cross reactivity will occur with antibody binding. As a result, it is important to determine the specificity of the mAb we obtain from the hybridoma cell line.

Our laboratory is not the first to create a hybridoma cell that secretes antibodies against asialo-GM1. The laboratory of Solomon and Higgins as well as that of Shimada and Iwata created hybrid cells which secrete antibodies against asialo-GM1. In the studies conducted by Solomon and Higgins, they used a similar immunization procedure to ours, except that their resting period lasted 185 days and our protocol called for two weeks resting (between injections). They isolated one clone which was secreting monoclonal antibodies

injections). They isolated one clone which was secreting monoclonal antibodies (determined by ELISA) against asialo-GM1. The isotype of the monoclonal antibody they isolated was of the IgM class (determined by ELISA). Using thin layer chromatography (TLC) and the ELISA test they analyzed the antibody for its specificity for several purified glycolipids (ie. Asialo-GM1, GM1 and asialo-GM3). From this they determined that the antibody was directed against the terminal, unsialylated galactose residue. When compared with commercially available rabbit polyclonal antibody, both the commerically available polyclonal and the monoclonal, cross reacted with GM1 and asialo-GM3 at high concentrations. They also tested the monoclonal antibody for the ability to disrupt the NK activity of spleen cells from Balb/c mice by incubating the spleen cells with the mAb and then adding complement. Complement is a group of serum proteins that is activated by the combination of an antigen with an antibody (in this case the mAb with the NK cell) resulting in the activation of a series of enzymes activation, resulting in cell lysis. In this experiment they discovered that all NK activity was removed because the interaction of the MAb with the NK cell activate the complement proteins and resulted in the lysis of the NK cells. In addition they tested the mAb on macrophages which express the asialo-GM1 molecule, and they were also lysed. This demonstrated that the mAb was specific for the antigen (asialo-GM1). Shimada and Iwata immunized various strains of mice with asialo-GM1 utilizing a similar method to Solomon and Higgins, except that the resting period was two weeks. Of the

strains tested, only the NZW mice gave a high anti-asialo-GM1 titer and these mice were used in the creation of hybridoma cells that secreted anti-asialo-GM1antibodies. In their studies, five clones were isolated that were secreting anti-asialo-GM1 monoclonal antibodies of the IgM class, as determined by the ELISA test. The monoclonal antibodies obtained were specific for only asialo-GM1 when tested by ELISA and Thin Layer Chromatography. They also tested the effect of these monoclonal antibodies on NK cell activity, and it was determined that complement was needed for the blockage of NK cell activity (same result as the studies by Solomon and Higgins). In addition they observed that the anti-NK activity of the NZW mouse antiserum was weaker than the rabbit polyclonal antibody. To get comparable results, a mixture of all monoclonal antibodies from all five clones had to be used. We hope that one of the mAb prepared in these studies will prove to be specific for the antigen asialo-GM1 following additional testing.

The ultimate goal of our studies is to use the monoclonal antibodies from our five clones to identify NK cells utilizing the immunostaining techniques described in 'material and methods'. As stated earlier, most studies with NK cells are done with some T cell contamination. To circumvent this problem, we hope to create a hybridoma NK cell which would provide us with a pure population of NK cells which can grow indefinitely in culture. The development of an NK hybridoma with NK activity is feasible, as one laboratory has already created a NK hybridoma. Suzuki and coworkers used spleen cells from a

Balb/c nude mouse, fused with a myeloma cell (NS-1), to create a NK cell hybrid. To confirm the physical and functional characteristics, these hybrids were analyzed to determine their surface phenotype and ability to lyse NK target cells. Identification of these cells was accomplished by immunolabeling the cells with ∝-Thy-1, ∝-CD4, ∝-CD8,∝ -CD25 (IL-2 receptor), or ∝-asialo-GM1 primary antibody, followed by a fluorescent secondary antibody and further analysis with a flow cytometry (an instrument use to measure the physical characteristics and fluorescent labeling of a cell). It was discovered that the hybrid NK cell exhibit asialo-GM1 and Thy-1 antigens with no markers for T cells. In addition, these cells lacked the expression of the IL-2 receptor. The authors thought this could be indicative of a resting NK cell. The hybrid NK cells were shown to lyse NK target cells but not negative controls.

We hope to use the monoclonal antibodies from the clones we have isolated to select for NK hybridomas in a pooled hybrid population. Once identified, we hope to have a pure population of cells that exhibit NK cell activity. These clones may be used as an investigative tool to better understand the mechanisms that control the functions of natural killer cells.

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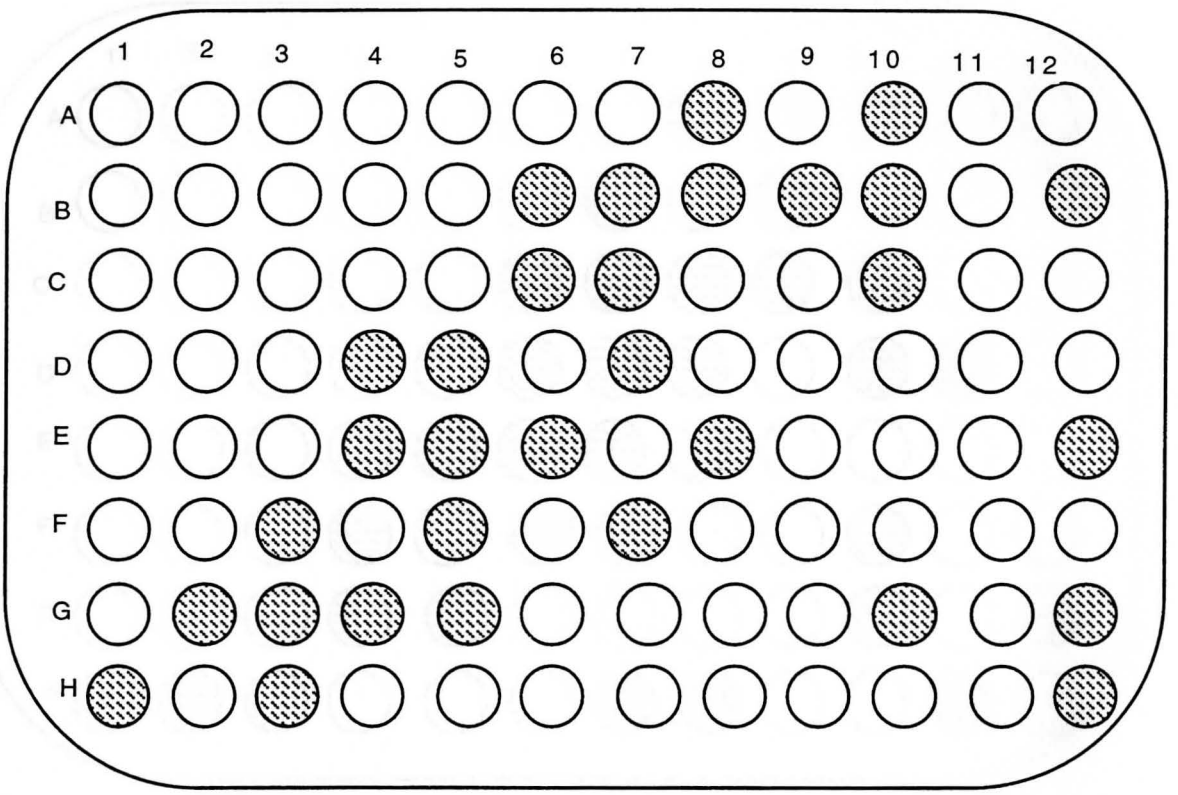
Appendix

Appendix A

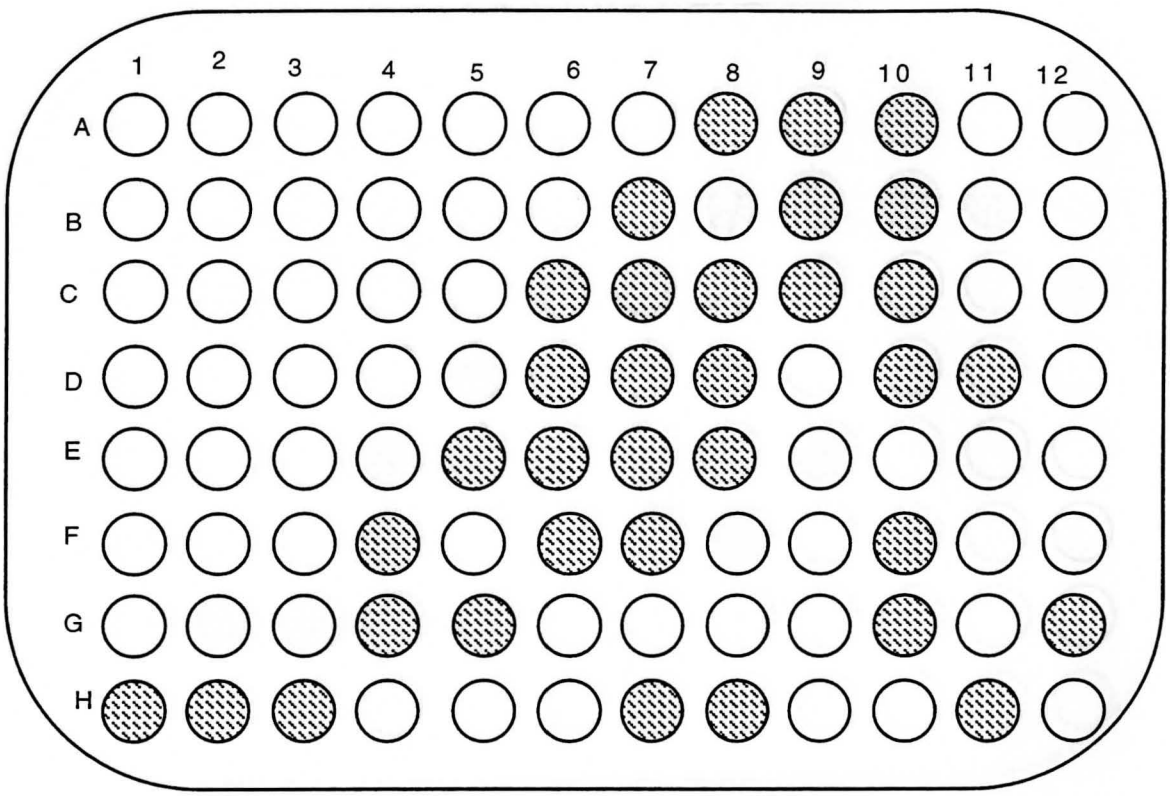
Subcloning of cells from A2, (3/8/96)



= Wells containing 1-5 clones

Appendix B

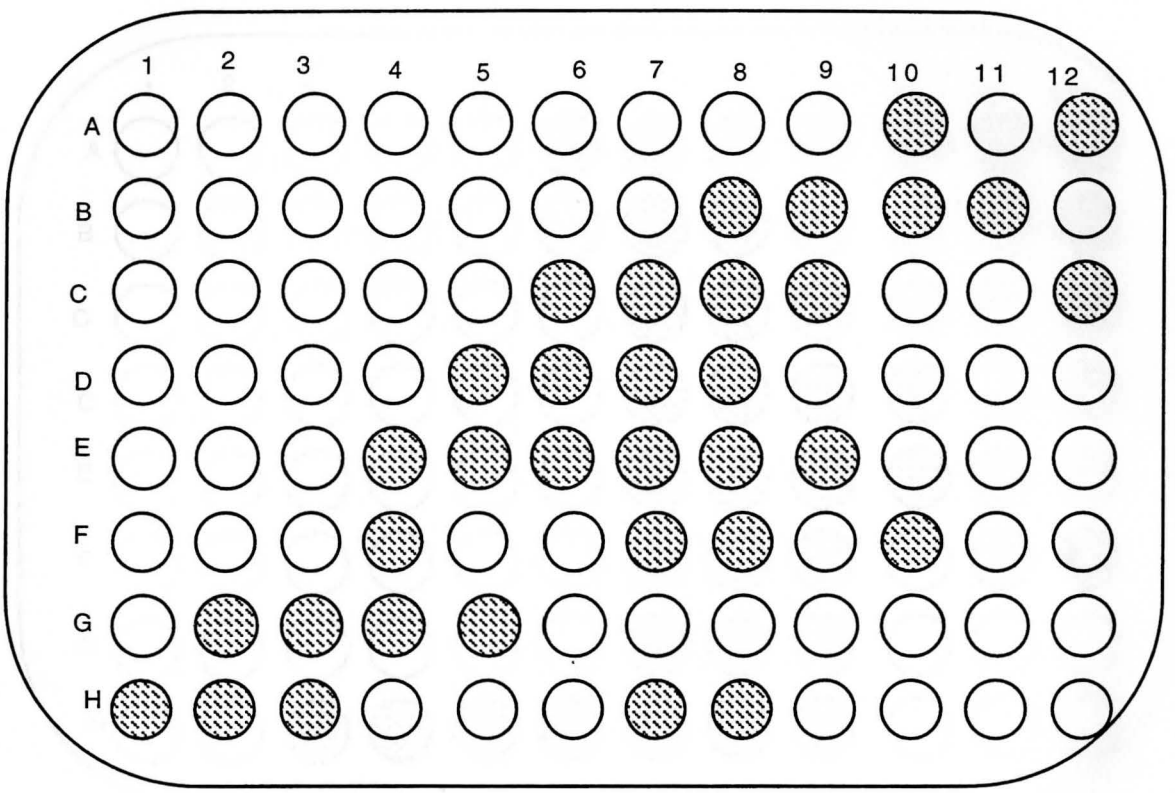
Subcloning of cells from A6, (3/8/96)



= Wells containing 1-5 clones

Appendix C

Subcloning of cells from B5, (3/8/96)



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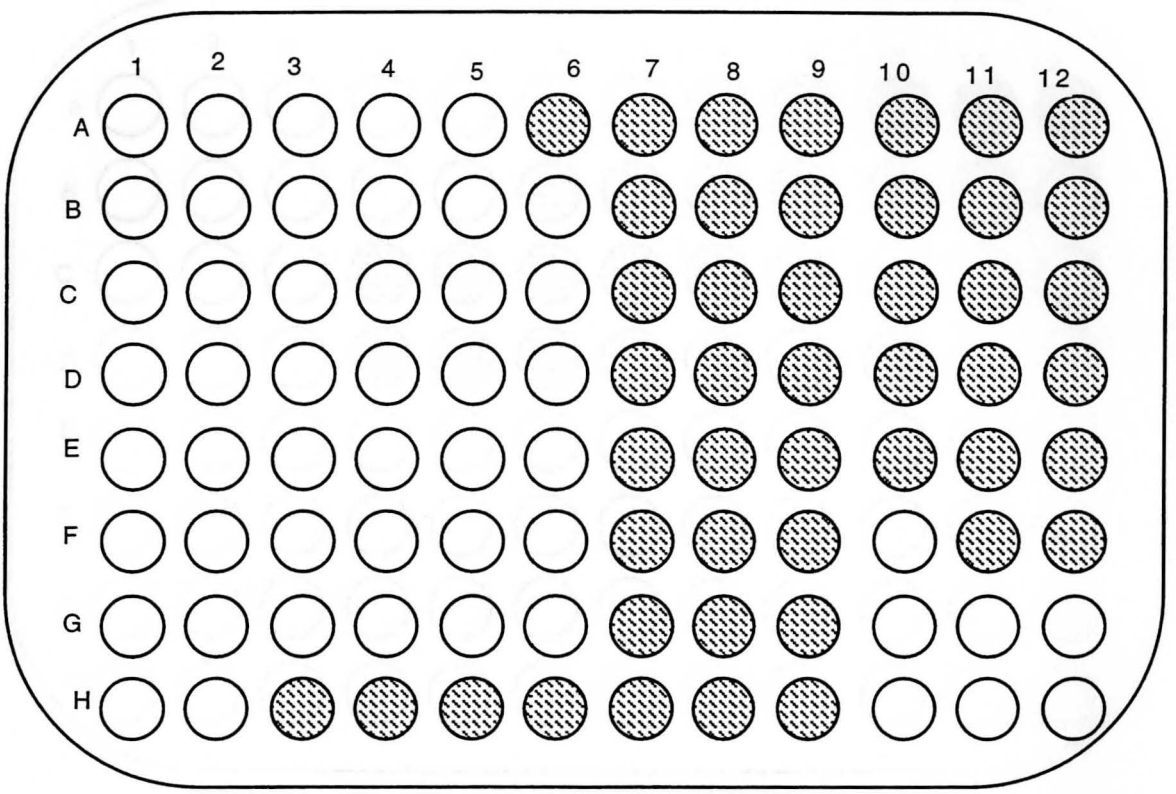
Appendix D

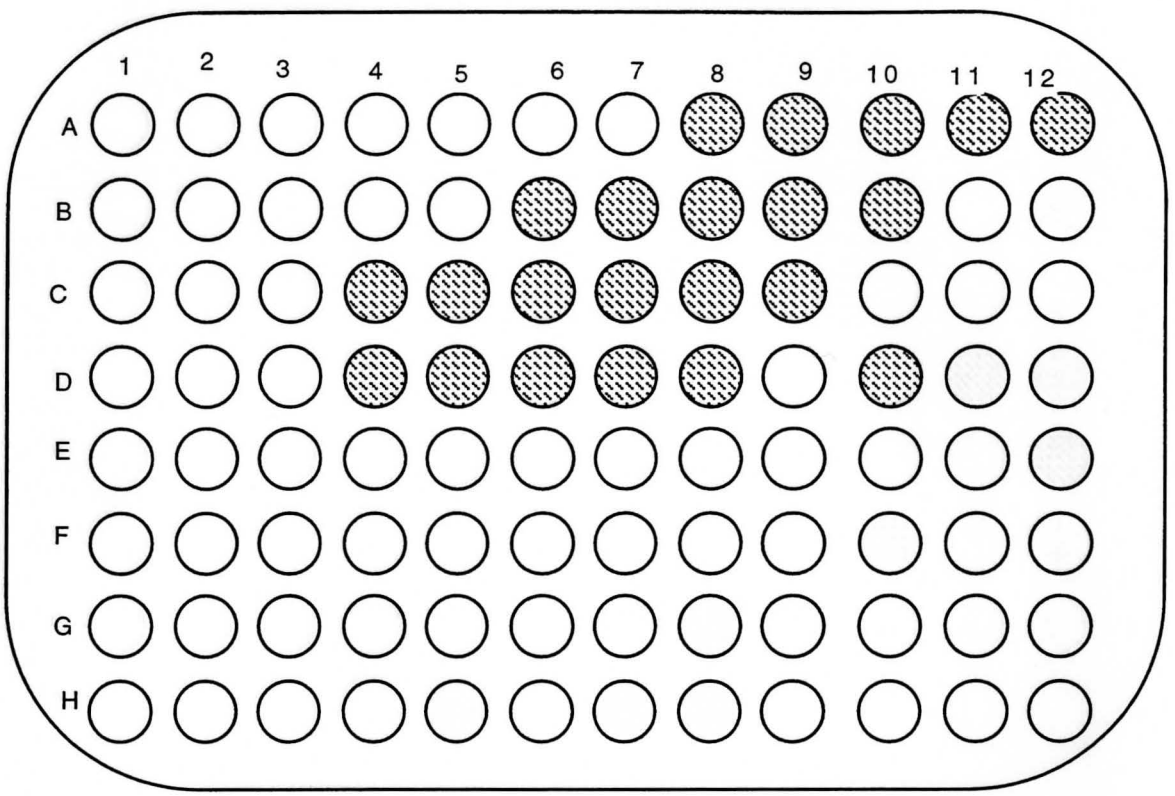
Sublconing of cell from C5, (3/8/96)



= Well containing 1-5 clones

Appendix E

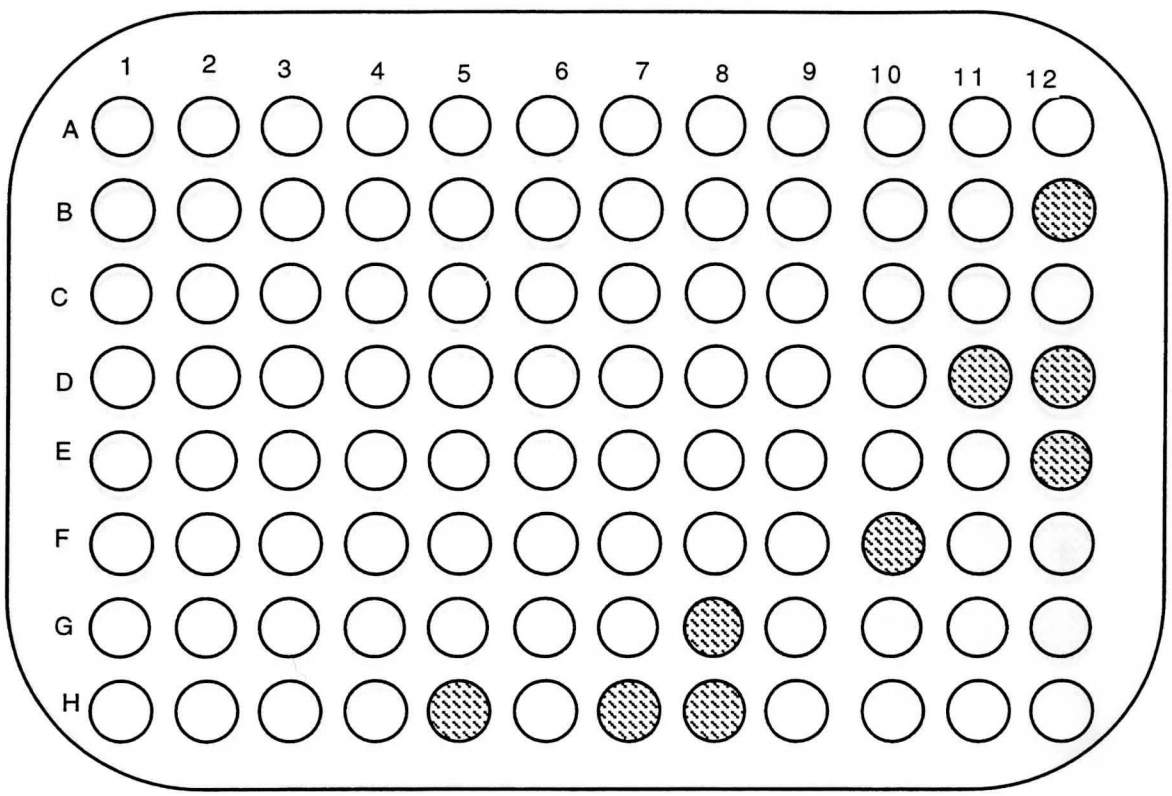
Subcloning of cells from D4, (3/8/96)



= Wells containing 1-5 clones

Appendix F

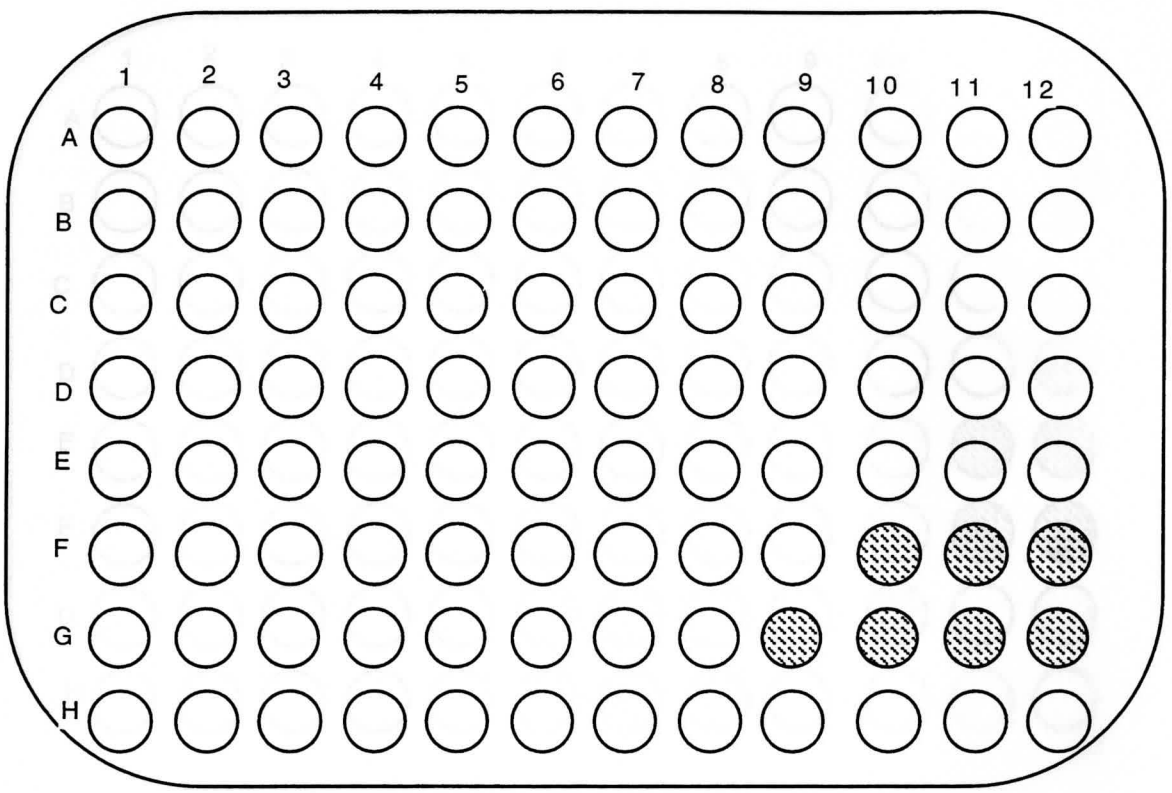
Subcloning of cells from HA2-B12



= Wells containing one clone

Appendix G

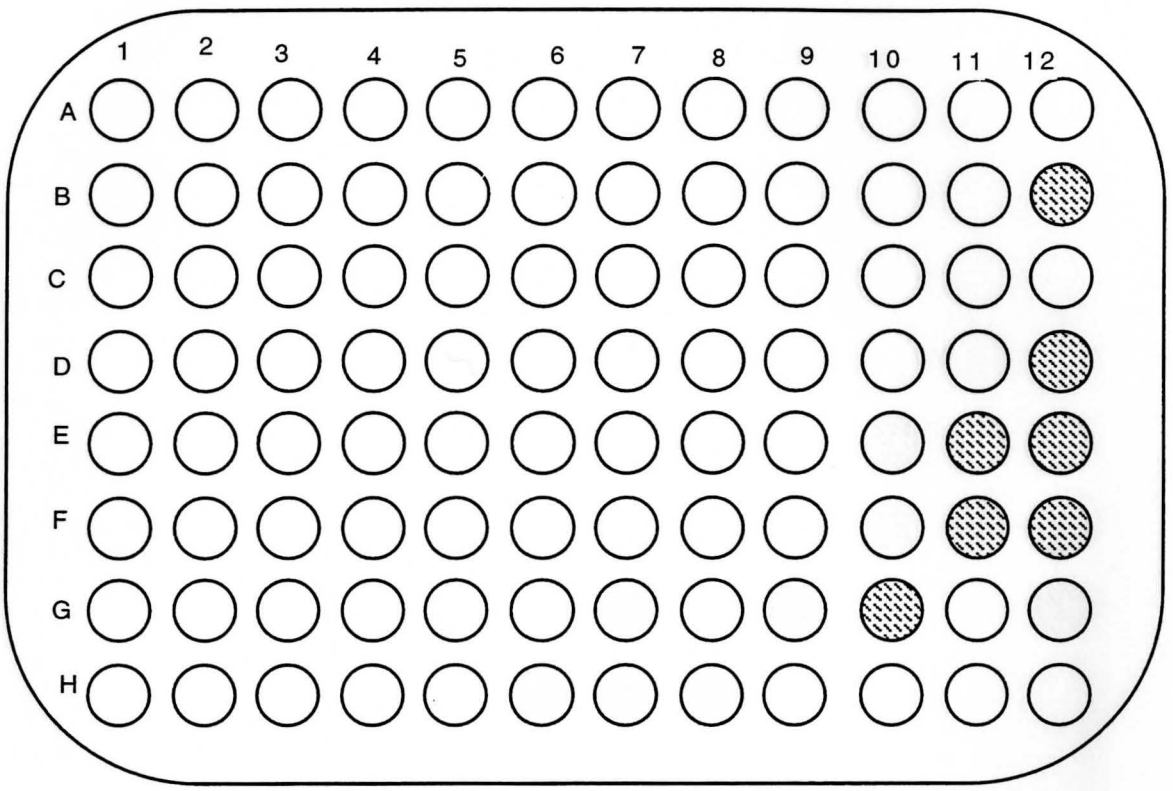
Subcloning of cells from HC5-B11



= Wells containing one clone

Appendix H

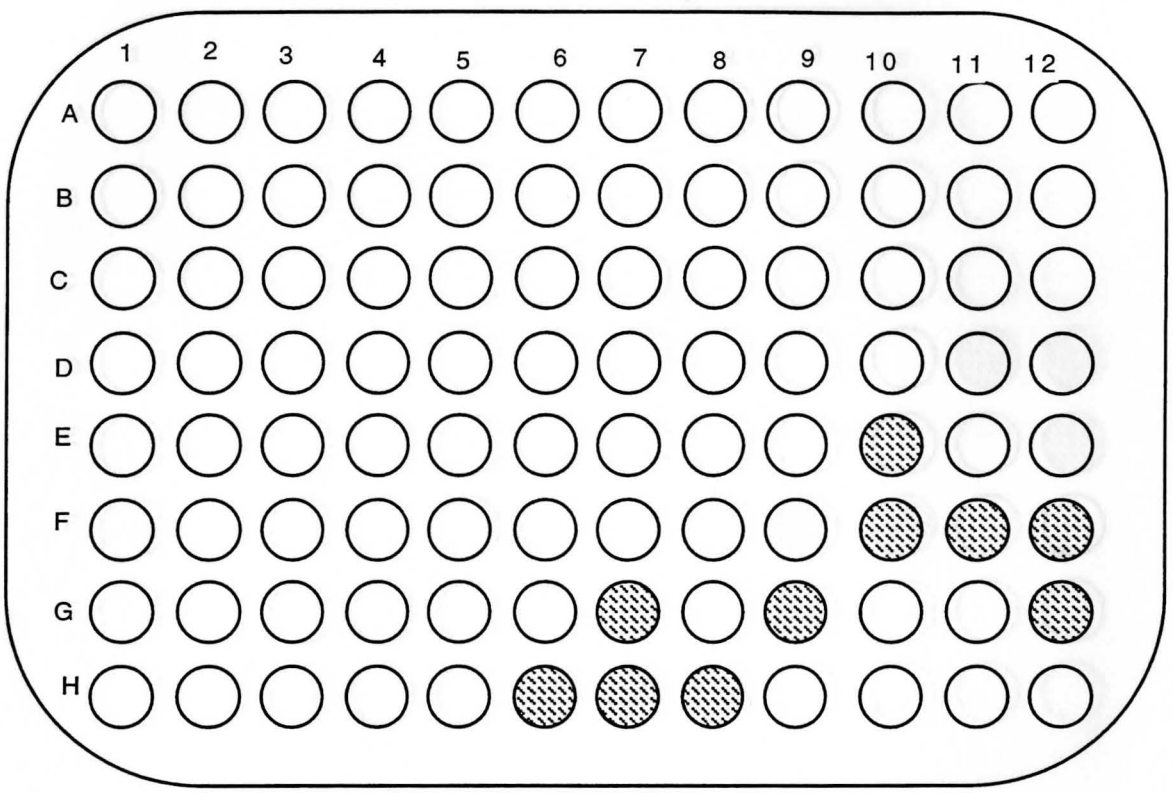
Subcloning of cells from HC5-B12



= Wells containing one clone

Appendix I

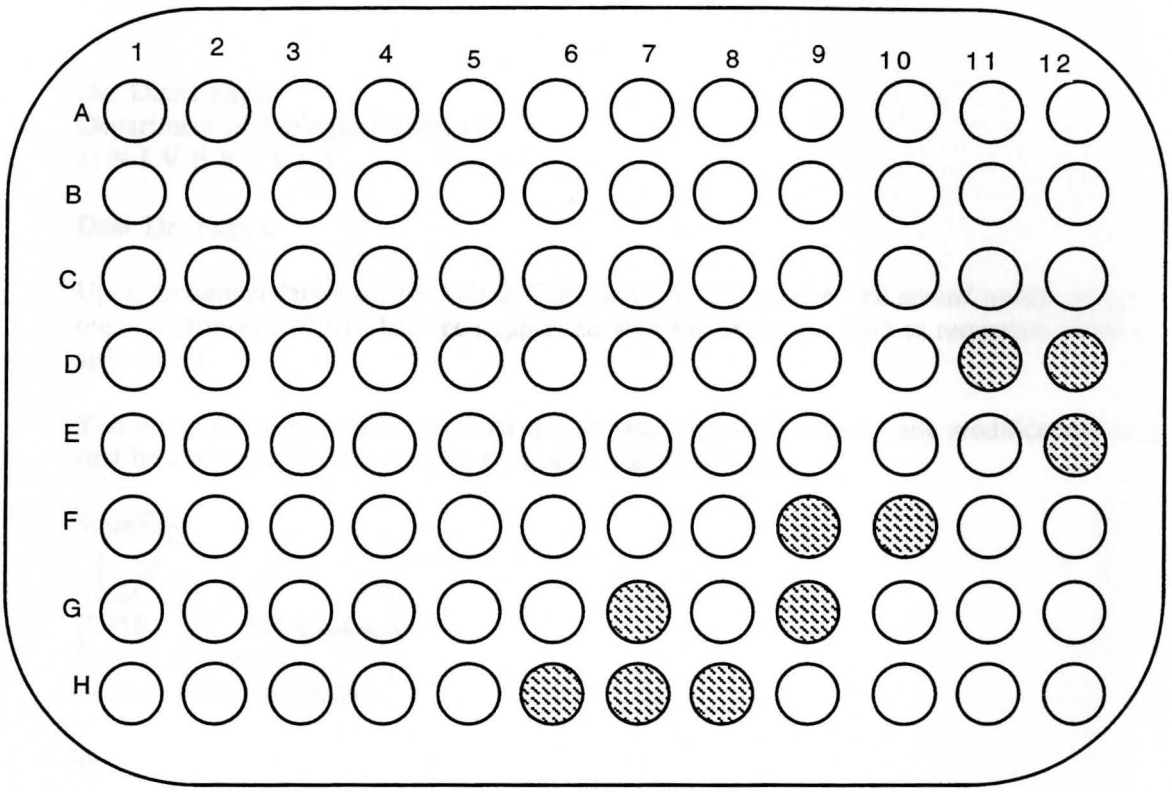
Subcloning of cells from HC5-C11



= Wells containing one clone

Appendix J

Subcloning of cells from HC5-D9



= Wells containing one clone

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Office of Grants and Sponsored Programs
(216) 742-2377

February 14, 1995

Dr. Diana Fagan
Department of Biological Sciences
U N I V E R S I T Y

Dear Dr. Fagan:

Upon recommendation of the Animal Care and Use Committee, the annual update of your ongoing protocol 92-016 has been approved with the expiration date as requested on your application.

You must adhere to procedures described in your approved request; any modifications must first be authorized by the Animal Care and Use Committee.

Sincerely,

Peter J. Kasvinsky
Dean of Graduate Studies

kb

Enclosure

c: Dr. Leipheimer, Chair, IACUC
Dr. Sobota, Chair, Biology