Production of Hybridoma Cells and Testing of Monoclonal Antibodies Directed Against Interleukin-12

by
Dara T. Beal

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Signature: Dara T. Beal 10/4/96
Student Date

Approvals: Nina Fagen 10/4/96
Thesis Advisor Date

Gary R. Walker 10/4/96
Committee Member Date

David Asch 10/4/96
Committee Member Date

Ruth J. Kanning 10/7/96
Dean of Graduate School Studies Date

ABSTRACT

Interleukin-12 is a cytokine that plays an important role is the regulation of the cell mediated immune responses. It is produced by macrophages and antigen presenting cells and causes the proliferation of natural killer cells and enhances their lytic ability. In order to further understand the effect of IL-12 on NK cells, a monoclonal antibody (a homogenous preparation of antibody molecules all exhibiting the same antigenic specificity) could be used to remove IL-12 activity. To prepare a monoclonal antibody against IL-12, a mouse was injected with interleukin-12 antigen. An enzyme-linked immunosorbant assay was used to measure antibody production. A hybridoma cell line was produced by the fusion of splenic B cells (antibody-producing cells) and P3X murine myeloma (cancer) cells. Fourteen viable hybridoma cultures E, F, G, H, I, J, K, L, M, N, O, P, Q, R were the result of this fusion. After confirming that all fourteen cultures were secreting antibodies against interleukin-12, one culture (G) was selected and subcloned to create the monoclonal antibodies H3, H4, H9, G6, G7, F7, F8, E9, D9. Subcloning involved isolation of a cell that would continuously divide to create a clone of genetically identical cells. Further confirmation of continual antibody secretion was done followed by a second subcloning of F8 and D9. The project was ended when the second subclone of F8 and D9 no longer secreted antibodies against interleukin-12. The remainder of the project will include a repeat cloning of the hybridomas producing monoclonal antibodies to obtain a twice cloned monoclonal antibody against interleukin-12. Further work will be needed to determine the isotype and specificity of the monoclonal antibodies produced as part of this study.

I thank Dr. Diana Fagan, Dr. David Asch and Dr. Gary Walker for comprising my thesis committee. I am fortunate and grateful for the opportunity to have worked under the supervision and advice of Dr. Fagan. I dedicate this thesis to my mother, Marjorie L. Beal, whose life and death made me the woman I am (2 Timothy 1:7). I thank all of my family and friends for their love, support, and words of encouragement. I thank **God** for being so good to me!

YSU

Youngstown State University / Youngstown, Ohio 44555-2377
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

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Antibody dependent cellular cytotoxicity (ADCC)

Cytotoxic T lymphocyte (CTL)

Enzyme-linked immunosorbant assay (ELISA)

Immunoglobulin (Ig)

Interferon (IFN)

Interleukin-12 (IL-12)

Lymphocytic choriomeningitis virus (LCMV)

Lymphokine-activated killer (LAK)

Monoclonal antibody (mAb)

Major histocompatibility complex (MHC)

Natural killer cells (NK)

Polyethylene glycol (PEG)

Tumor necrosis factor (TNF)

INTRODUCTION

The immune system is the adaptive defense system that has evolved in vertebrates to recognize and protect them from invading pathogens. Immunity is the state of protection from infectious disease, and it has both nonspecific and specific components. Innate nonspecific immunity involves basic defenses such as anatomic and physiologic barriers, white blood cell phagocytosis, and inflammation. Phagocytosis involves the ingestion of extracellular macromolecules and delivery to lysosomes. The degradative enzymes within the lysosomes digest the macromolecules to small breakdown products that can be eliminated from the cell. The inflammatory response is a localized response to tissue damage caused by a wound or by invasion of a pathogenic microorganism. It is characterized by vasodilation, increased capillary permeability, and influx of phagocytic cells.

Acquired specific immunity includes specific recognition and selective elimination of foreign microorganisms and molecules. The specificity of the immune system allows discrimination of subtle differences among antigens. The immune system can generate tremendous diversity in its recognition of molecules, allowing it to specifically recognize a vast number of uniquely different structures on foreign antigens. Memory is a heightened state of immune reactivity to an antigen that has previously been encountered. This confers life long immunity to many infectious agents. The immune system is capable of distinguishing self from nonself. This is essential for responding only to nonself antigens, because inappropriate responses to self antigens may result in

autoimmune disease. Specificity, diversity, memory, and self/nonself recognition are all critical aspects of the acquired immune system.

One branch of acquired immunity is known as the humoral immune response. The fundamental cell of the humoral branch is the B lymphocyte. B cells mature in the bone marrow and have antibody molecules bound to their membrane. When a naive B cell encounters antigen for the first time, it divides rapidly into memory B cells and plasma cells. Memory B cells continue to express particular membrane-bound antibody, and they mediate a faster and greater secondary response. Plasma cells are effector cells that secrete antibodies that recognize an epitope on the antigen and facilitate clearance of that antigen.

T lymphocytes comprise the cell-mediated branch of acquired immunity. They arise from hematopoietic stem cells in the bone marrow, and they migrate to the thymus gland to mature. The antigen-binding receptor of T lymphocytes is known as the T-cell receptor. T-cell receptors can only recognize antigen in association with major histocompatibility complex (MHC) molecules. These molecules are required for antigen presentation to T cells and for rapid graft rejection. After a naive T cell has encountered antigen associated with an MHC molecule, it differentiates into memory T cells, T helper cells, and T cytotoxic cells. Memory T cells mediate a faster and greater secondary response. T helper cells express CD4+ glycoproteins on their membranes and respond to recognition of antigen-MHC complexes by secreting cytokines. Cytokines are low-molecular weight proteins that regulate the intensity and duration of the immune response. This is facilitated by exerting a variety of effects on T cytotoxic cells, natural killer

cells, and other immune cells. T cytotoxic cells express CD8+ glycoprotein on the cell membrane and respond to recognition of antigen-MHC complexes by differentiating into cytotoxic T lymphocytes. These are effector cells that exhibit cytotoxic activity and eliminate virus-infected cells, tumor cells, and cells of a foreign tissue graft.

Another lymphocyte of particular interest to this research project is the natural killer (NK) cell. It is a large granular lymphocyte that has cytotoxic ability, but does not express antigen-binding receptors nor does it require MHC. NK cells exhibit nonspecific cytotoxicity against tumors and virus-infected cells in a manner similar to CTL-mediated lysis. After an NK cell adheres to a target cell, degranulation of perforin-containing granules occurs. This release of perforin damages the target cell. NK cells may also destroy target cells by stimulating apoptosis, which is programmed cell death (1).

An editorial written by Moretta et al. entitled *Origin and Functions of Human Natural Killer Cells*, genetically analyzes the mode of inheritance of target cells in their susceptibility or resistance to lysis by NK cells (2). Their observations revealed that the expression of given HLA class I alleleles protects target cells from lysis by different groups of natural killer clones. The findings of their experiment were consistent with the hypothesis that NK cells recognize either polymorphic epitopes of class I molecules or structures selectively bound to certain HLA alleles. Their data showed that a loss of defined HLA-C alleles rendered the cell variants susceptible to lysis by defined groups of alloreactive clones. Cell transfectants for different HLA class I alleles or cell variants expressing only single class I alleles provided resistance to lysis. The expression on target cells of the HLA-Cw4 allele rendered the cell

resistant to lysis by an arbitrarily named group 1 NK clone. The HLA-Cw3 allele also rendered the cell resistant to lysis by group 2 NK clone. They believed resistance to lysis was dominantly inherited and co-segregated with HLA haplotypes. Any pathological event which would cause lack or masking of expression, such as tumor transformation or viral infection, would render the cells susceptible to lysis by NK cells.

An article written by Yuan et al. explained the interactions between B lymphocytes and natural killer cells (3). Evidence has accumulated to show that NK and B cells interact productively. They found that NK cells secrete a factor (or factors) that activate, in vivo, preactivated B lymphocytes directly. The nature of the factor did not appear to be a lymphokine known to be produced by NK cells. Because the factor is labile, they believed that cloning its gene would be difficult. NK cells produce a second factor that can induce B cells to secrete polyclonal Ig. This is mediated by a soluble factor or factors that appears to be different from any known cytokine. The in vivo relevance of these interactions revealed that activated NK cells can increase the IgG2a response to a specific protein antigen. They also learned that B lymphocytes may exert an effect on NK cells. Preactivated B lymphocytes can induce NK cells to produce greater amounts of IFN-gamma via an interaction that requires direct cell contact. Interferon (IFN) refers to several glycoproteins produced and secreted by certain cells that induce an antiviral state in other cells and also helps to regulate the immune response. IFN-alpha and IFN-beta primarily provide antiviral protection whereas IFN-gamma which is produced by T cells, has numerous effects on various immune-system cells (1). Their study showed that NK

cells did not have the ability to kill primary B lymphocytes, as previously speculated, regardless of their stage of differentiation.

Various cytokines have immunoregulatory effects on natural killer cells. Cytokines are any of numerous secreted, low-molecular weight proteins that regulate the intensity and duration of the immune response by exerting a variety of effects on lymphocytes and other immune cells (1). Interferons, interleukin-2 (IL-2), interleukin-7, interleukin-12 and interleukin-6 were all shown to be responsible for direct activation of NK cells (4). IL-2 has been shown to potentiate NK activity, increase the cytotoxic granule content of NK cells, and enhance adhesion molecule expression. It induces lymphokine-activated killer (LAK) cell phenomenon [LAK refers to a heterogeneous population of cytotoxic cells (1)] and proliferative activity in human NK cells. IL-2 increases antibody dependent cellular cytotoxicity (ADCC). ADCC refers to NK cells or other nonspecific cytotoxic cells that express Fc receptors recognizing bound antibody on a target cell and subsequently causing lysis of the target cell (1). IL-2 induces mRNA transcription and secretion of cytokines. When IFN-alpha or IFN-gamma are used as co-stimulus to IL-2, there was an enhancement of the IL-2 induced NK cytotoxicity. Longer co-culture with IFN-gamma and IL-2 reduces the lytic activity compared to the effects of IL-2 alone. The inhibitory effect may be mediated through adherent accessory cells. The interferons did not cause NK cell proliferation and they inhibited the proliferative activity induced by IL-2 in peripheral blood mononuclear cells (4).

IL-7 induces substantial levels of LAK activity in purified CD56+ NK cells in 5 days culture. CD56+ is a cell membrane molecule of NK cells that functions in adhesion and it is identified by a monoclonal antibody

(3). However, IL-7 induced an 8-15 fold lower proliferative activity in CD56+ NK cells than IL-2. IL-7 mixed with a suboptimal concentration of IL-2 potentiated the proliferation of NK cells over that seen with IL-2 alone (4).

IL-6 is thought to modulate NK cell functions in mononuclear cell populations (4). Peripheral blood mononuclear cells cultured with IL-6 showed increased NK cell cytotoxicity. This effect was mediated through an endogenous production of IL-2. IL-6 also caused the induction of adhesion molecules, TNF-gamma and TNF-beta cytokine production, and it caused low levels of proliferation in purified $CD3^{-}CK56+$ NK cells.

NK cells are able to secrete several cytokines, but the presence of contaminating cells in the NK population interferes with interpretation of the data (4). IFN-gamma can be produced from highly purified NK cell cultures. NK cells can secrete TNF in response to IL-2, IL-6, IL-7, IL-12 or FcR-interactions. IL-2-activated NK cells may also secrete TGF-beta and IL-8. The ability of natural killer cells to secrete various cytokines indicates they may function to modulate the activities of other leukocytes or leukocyte precursors.

IL-12 is a cytokine of particular interest in my research project. It is produced by macrophages and B lymphocytes. It effects T lymphocytes and natural killer cells by stimulating cytotoxic activity and proliferation. IL-12 promotes the development of T cells that are responsible for delayed type hypersensitivity and induces the production of IFN-gamma and TNF (5). IL-12 was shown to increase NK activity and to increase levels of IFN-gamma production in NK cells and activated T cells. When IL-12 was added to cultures of purified resting NK cells, there were high levels of NK cytotoxicity (5). The effect of IL-12 was independent of IL-2.

LAK activity induced by IL-12 was approximately 50% of that induced by IL-2. The maximum enhancement of NK cytolytic activity by IL-12 is achieved at picomolar concentrations, while IL-2 requires nanomolar concentrations. IL-12 also caused an enhancement of ADCC, cell-adhesion molecule expression, and increased NK cell granule contents. Low IL-12-induced proliferative activity was recorded but IL-12 potentiated the proliferative activity of NK cells stimulated with suboptimal levels of IL-2 (4).

IL-12 has many functions (6). In short term incubations of in vitro systems, IL-12 stimulated NK cells to kill NK-sensitive cells, NK-resistant cells, antibody-coated tumor target cells, and virus-infected fibroblasts and T cells. The effects of IL-12 were shown to function independent of IL-2. The addition of anti-IL-2 antibodies, anti-IL-2 receptor antibodies and IL-4 (all inhibit NK activity induced by IL-2) did not inhibit IL-12 activation of NK cells.

IL-12 has been shown to influence the proliferation of T cells and NK cells in various in vitro assays (6). IL-12 does not stimulate proliferation in resting peripheral blood mononuclear cells, but it does induce proliferation of lymphocytes that have been previously activated by lectins, phorbol esters, IL-2, anti-CD3, or allogeneic stimulation.

When NK cells are purified away from other contaminating cell types, IL-12 can induce the proliferation of NK cells. When CD56+ NK cells were stimulated with IL-12, the level of induced proliferation was only 10% of the level induced by IL-2 and 50% of the IL-7-induced proliferative response. Depending on the conditions used, IL-12 had either an additive or inhibitory effect on IL-2-induced NK cell proliferation. Dosage was a factor influencing this proliferation. Where low doses of IL-2 was

added, IL-12 enhanced proliferation. Conversely, at higher doses IL-2 inhibition was observed by the addition of IL-12. It was also learned that combinations of IL-4 and IL-12 resulted in synergistic proliferation of NK cells (6).

IL-12 has been tested clinically and shown to be useful in the treatment of parasitic infections, viral diseased, and malignancies (6). Murine leishmaniasis is a disease associated with the expansion of CD4+ T cell subsets. CD4+ molecules are the adhesion molecules that bind to class II MHC molecules and also function in signal transduction (1). It was discovered that IL-12 could be used for treatment because it is able to stimulate IFN-gamma synthesis, a cytokine that inhibits the parasite. IL-12 is also able to augment the development of CD4+T_H1 cells (T cells responsible for a cellular response to parasites) which prevents the production of IL-4. The proposed mechanism is thought to occur as follows. First IL-12 stimulates NK cells to produce IFN-gamma, which in turn activates macrophages to exhibit antileishmanial activity. IL-4 down regulates this macrophage activation but the induction of T_H1 cells results in lowered amounts of IL-4.

Mice injected with lymphocytic choriomeningitis virus (LCMV) develop a strong protective CD8+ cytotoxic T lymphocyte response (6). Treatment with IL-12 decreased replication of the virus and enhanced the number of CD8+ T cells in the lymph nodes, spleen, and blood. Synergistically elevated levels of serum IFN-gamma and TNF were found in mice receiving IL-12 treatment. The mechanism involved in stimulation of the cytotoxic T cell response is unclear, but it is presumed that IL-12 causes an increase in IFN-gamma and TNF and these both inhibit the virus.

IL-12 also has antimetastic and antitumor activity in a number of murine tumor models (6). Experimentally-induced pulmonary metastases of the B16F10 melanoma showed antimetastic effects with dose dependent IL-12 treatment. IL-12 treatment reduced experimentally-induced hepatic metastases of M5076 reticulum cell sarcoma and increased survival. Activity against established subcutaneous tumors was observed after IL-12 treatment. Renca renal cell carcinoma, B16F10, M5075, and several other tumors responded to IL-12 treatment. The observed activity against the established subcutaneous tumors included substantial growth inhibition, prolongation of survival, rejection of subsequent rechallenge with the same tumor, and in some instances complete tumor regression.

Because treatment with high doses of cytokines often results in toxicity, clinical chemistry and hematology parameters were evaluated. Leucopenia (a decrease in both circulating neutrophils and lymphocytes), moderate increases in hepatic transaminases, and anemia were all observed in IL-12 treated mice (6). However, lower therapeutic doses of IL-12 caused relatively few toxicities (6).

A number of experiments have been performed to elucidate the mechanism of IL-12 effect on tumor cell growth. The following has been proposed. First IL-12 induces cytokine secretion from NK cells and/or T cells. IFN-gamma is one of the cytokines secreted and it is critical for antitumor efficacy. IFN-gamma can activate macrophages present in the tumor and can contribute to the development of CD8+ cytolytic T cells. The cytolytic T cells inhibit the growth and/or induce regression of the tumor (6). Other cytokines produced may also contribute to anti-tumor efficacy (6).

Because IL-12 is a vital cytokine, a monoclonal antibody directed against it would be a useful research tool. A monoclonal antibody is a homogenous preparation of antibody molecules all exhibiting the same antigenic specificity. Monoclonal antibodies are produced by cloned hybridoma cells that secrete antibody, like a B cell, and live indefinitely in culture, like a myeloma cell (7). The production of a hybridoma cell line involves the fusion of myeloma cells (cancerous plasma cells) with B cells from the spleen of a mouse immunized with the antigen IL-12. When cells are treated with Sendai virus or high concentrations of polyethylene glycol (PEG) their membranes fuse. The resulting cell is known as a heterokaryon, a multinucleated cell. At the next cell division, the nuclei of the heterokaryons fuse, and the daughter cells posess a more or less equal share of the genetic material. The hybrid cells are genetically unstable and a loss of chromosomes occurs. This loss of chromosomes is thought to be preferential depending on the species and the cell type. When a hybridoma cell is isolated in culture, it will divide to produce a clone of genetically identical cells and these cells will produce the monoclonal antibodies (8).

Monoclonal antibodies are useful in research. They can be used to purify proteins (1). A monoclonal antibody specific for the protein of interest is attached to beads to form an immunoadsorbent column. The crude preparation is passed through the column and the protein of interest may then be eluted. Monoclonal antibodies can identify and isolate lymphocyte subpopulations and clones (1). If monoclonal antibodies to CD4+ and CD8+ are labeled with two different fluorochromes and then incubated with a lymphocyte preparation, an investigator would be able to separate the T helper cells from the T

cytotoxic cells with a fluorescence activated cell sorter. Monoclonal antibodies can be used for tumor detection and imaging (1). Tumor-associated membrane proteins are absent or at low levels on normal cells but are present on tumor cells. When a monoclonal antibody specific for a certain tumor-associated membrane protein is created, the tumor can be detected. Radiolabeled monoclonal antibodies can be used to image primary or metastic tumors in patients. Tumor killing can be facilitated by monoclonal antibodies and complement-mediated lysis (1).

In summary, the activities of IL-12 suggest that it may play a role in the defense against intracellular microbial infections and cancer by enhancing the cytolytic function of NK cells. A monoclonal antibody directed against IL-12 would be of particular interest because it could be used in bioassays examining the effect of IL-12 on NK cell function. In the following studies, a monoclonal antibody directed against mouse IL-12 will be prepared and tested by ELISA for the ability to bind to IL-12.

MATERIALS

Reagents

IL-12 was purchased from R&D Systems, Inc. (Minneapolis, MN). Freund's adjuvant, cryogenic vials, oxaloacetate, pyruvate, insulin, hypoxanthine, aminopterin, thymidine, polyethylene glycol, mouse serum, O-phenylenediamine, sodium bicarbonate (for cell culture use), sodium phosphate, Tris, EDTA, Tween-20, and hydrogen peroxide were all purchased from Sigma Chemical Company (St. Louis, MO). The 96-well plates used in the experiment were purchased from Becton Dickinson Labware (Oxnard, CA). The 24-well plates used in the experiment were purchased from Corning Glass Works (Corning, NY). The cryogenic vials used to freeze aliquots of cells were purchased from Sigma Chemical Company (St. Louis, MO). The MRC-5 fibroblast cells (passage 5) and the P3X63AG8.653 myeloma cells were both purchased from American Type Culture Collection (Rockville, MD). RPMI1640, fetal calf serum, penicillin/streptomycin, and L-glutamine were all purchased from Life Technologies (Grand Island, NY). The secondary antibody of the ELISA was goat anti-mouse IgG+IgA+IgM (H+L) peroxidase, purchased from Life Technologies (Gaithersburg, MD). Sodium chloride was purchased from Fisher Scientific (Fair Lawn, NJ).

METHODS

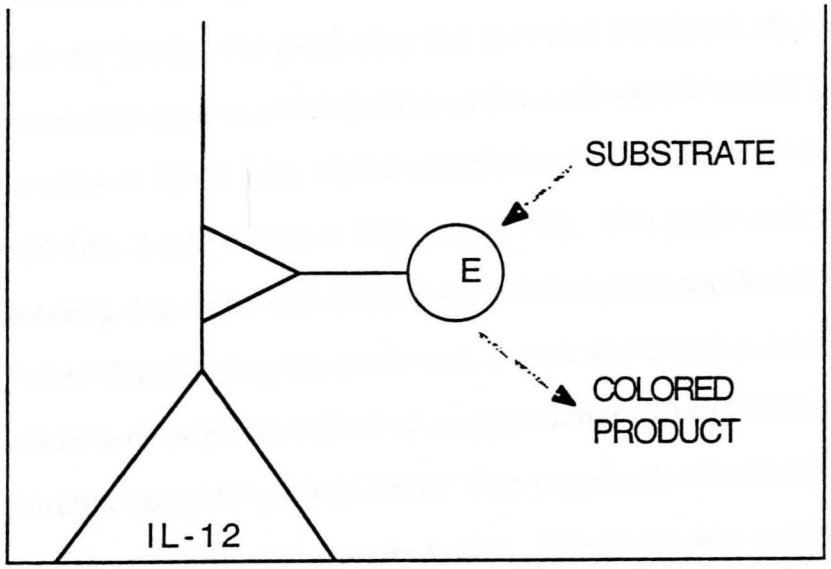
Mouse vaccination

A DBA-2 strain mouse was purchased from Jackson Laboratories. It was immunized with 5 ug of IL-12 dispersed in 250 ul of sodium chloride (saline). This solution was drawn into a 1.0 ml syringe and mixed with an equal amount of complete Freund's adjuvant. The solution was injected into the mouse intraperitoneally. Seven days later 5 ug IL-12 dispersed in 250 ul of sodium chloride (saline) mixed with an equal amount of incomplete Freund's adjuvant was injected intraperitoneally. One week later blood was collected from the mouse and tested by ELISA (see below) for the presence of antibodies directed against IL-12. As IL-12 antibodies were detected, the cell fusion could be done. To assure development of plasma cells, an intravenous booster of 5 ug of IL-12 in 250 ml of saline was administered three days prior to the collection of spleen cells for the cell fusion (9).

ELISA

The ELISA used throughout this study is based on antibody present in the mouse serum or cell culture supernatant binding to IL-12 coating the bottom of a microtiter plate well (see Figure 1). A secondary antibody (from a goat immunized with mouse antibodies) is conjugated with an enzyme (horseradish peroxidase), and will bind to the antibody of interest. When substrate (OPD-orthophenylenediamide) is added, it reacts with the enzyme to yield a colored product. The whole well appears colored and the 96-well plate can be read on a Microplate Autoreader (model EL311 Bio-Tek Instruments) at 490 nm. Conversely, a negative well will have no antibody present to bind the adherent IL-12. The secondary antibody conjugated with an enzyme will have no antibody to bind, and no colored product is obtained. Blood was collected from the capillaries behind the eye of an anesthetized mouse. The serum was

Figure 1. ELISA For Interleukin-12. The top well reprsents a positive result and the bottom well represents a negative result.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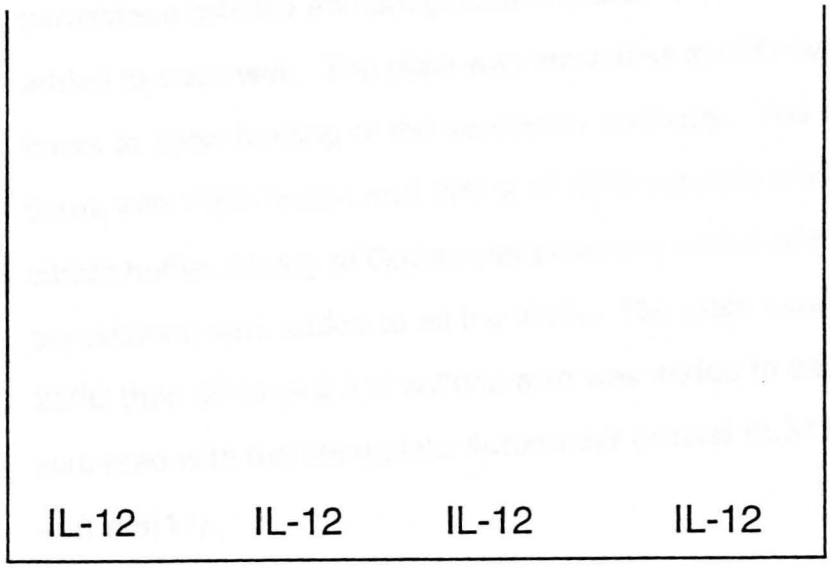
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isolated and refrigerated. A 96-well plate was coated with 100 ul of IL-12 (1ug of IL-12 brought up to 2 ml in 0.5 mM sodium carbonate buffer, pH 9.6) and incubated overnight at 4°C (see Figure 1). The solution was removed from the plate by flicking the plate over the sink and blotting it on a paper towel. The unreacted protein binding sites of the well were blocked by incubating for 30 minutes at 22°C with 150 ul of blocking buffer (1% BSA in 150 mM NaCl, 50 mM Tris, 2 mM EDTA in TBS, at pH 7.4). The plate was washed three times by flooding the wells with PBS-Tween (phosphate buffered saline with 0.05% Tween-20), flicking the plate over a sink and blotting it on a paper towel. The blank well received 100 ul of sample buffer (50 mg BSA and 5 ul Tween-20 brought up to 10 ml with TBS). The negative control well received 1 ul of mouse serum and 100 ul of sample buffer. The plate was incubated at 22°C for one hour or 4°C overnight to allow antibody binding. The wells were washed three times with PBS-Tween and 100 ul of goat anti-mouse IgG+IgA+IgM (H+L) peroxidase labeled immunoglobulin, diluted 1:1000 in sample buffer, was added to each well. The plate was incubated at 4°C overnight or 22°C for two hours to allow binding of the secondary antibody. The plate was washed three times with PBS-Tween and 100 ul of OPD solution (10 ml 0.1M phosphate citrate buffer, 10 mg of O-phenylenediamine, and 4 ul of 30% hydrogen peroxidase) was added to all the wells. The plate was incubated 30 minutes at 22°C then 50 ul of 2.5 N sulfuric acid was added to each well. The absorbance was read with the Microplate Autoreader (model EL311 Bio-Tek Instruments) at 490 nm(11).

Cell fusion

P3X myeloma cells grown in RPMI were incubated with OPI media the morning of the fusion (9). OPI media consisted of RPMI supplemented 1:1000 with 1000X OPI (1.5 g oxaloacetate, 500 mg sodium pyruvate, 2000 IU bovine insulin in 100 ml H_2O) (9). The vaccinated mouse was anesthetized by metaphane inhalation. Blood was drawn from the heart and the spleen was removed. The spleen cells were isolated under sterile conditions by pressing the spleen through a sieve into complete RPMI [RPMI enriched with 20% heat inactivated (65°C for 45 minutes) fetal calf serum, 1% penecillin/streptomycin (10,000 units/ml of each in 0.85% saline), and 1% L-glutamine (29.2 mg/ml in 0.85% saline)]. The solution was allowed to sit for five minutes to allow clumps to settle, then the supernatant was removed. The supernatant was centrifuged at 300 x g for eight minutes. Tris buffered ammonium chloride (90 ml 0.16M NH_4Cl and 10 ml 0.17M Tris pH 7.65 and adjusted to pH 7.2 with HCL) was added to the pellet to lyse the red blood cells (10). The cells were underlain with 3 ml of fetal calf serum and spun at 300 x g for 10 minutes. Media (10 ml) was added to the spleen cell pellet and the cells were counted on a hemocytometer. The fusion was performed according to the method described by Harlow and Lane in *Antibodies: A Laboratory Manual* (10). The myeloma cells, which live indefinitely in culture and are $HGPRT^-$, were counted and a solution was made containing 5 spleen cells to 1 myeloma cell. The cells were combined in a 50 ml conical tube and washed twice by adding fresh media without fetal calf serum and centrifuging at 200 x g for 8 minutes. The supernatant was removed and 1 ml of 50% polyethene glycol was added dropwise to the cell pellet over one minute while shaking the test tube. RPMI without fetal calf serum (1 ml) was then added dropwise over one minute while shaking the test tube. An additional 8 ml of media was added dropwise over five minutes while shaking the test tube, and the cells brought up to 50 ml with

RPMI without fetal calf serum. The tube was centrifuged at 200 x g for eight minutes. The cell pellet was resuspended in 24 ml of media. The fused cell were then dispensed (1ml/well) into a 24-well plate containing MRC-5 cells. The feeder layer cells (MRC-5) used in this project were fed DMEM media supplemented with 10% heat inactivated (65^{o}C for 45 minutes) fetal calf serum, 1% penecillin/streptomycin (10,000 units/ml of each in 0.85% saline), and 1% L-glutamine (29.2 mg/ml in 0.85% saline). The supernatant was aspirated from the MRC-5 cells before the addition of the hybridoma cells (B cells fused to a cancer cell). The MRC-5 fibroblasts function by secreting unidentifiable cell to cell factors that enable hybridomas to survive harsh adverse conditions. Fibroblasts are also useful because they adhere to the culture well or flask, while the hybridomas remain in suspension in the media (8). The cells were then incubated at 37^{o}C and 5% CO_2. After 24 hours, 1 ml of media containing 2X HAT was added to each well. HAT media consisted of complete RPMI supplemented with 50X HAT (when reconstituted in 10 ml each vial will contain 5 x 10-3M hypoxanthine, 2 x 10^{-5}M aminopterin, 8 x 10^{-4}M thymidine). After 14 days HAT was left out of the media and HT supplemented media was used for 7 days. HT media consisted complete RPMI supplemented with 50X hypoxanthine and 50X thymidine (when reconstituted in 10 ml each vial will contain 5 x 10^{-3}M hypoxanthine and 8 x 10^{-4}M thymidine) (8).

Cell maintenance

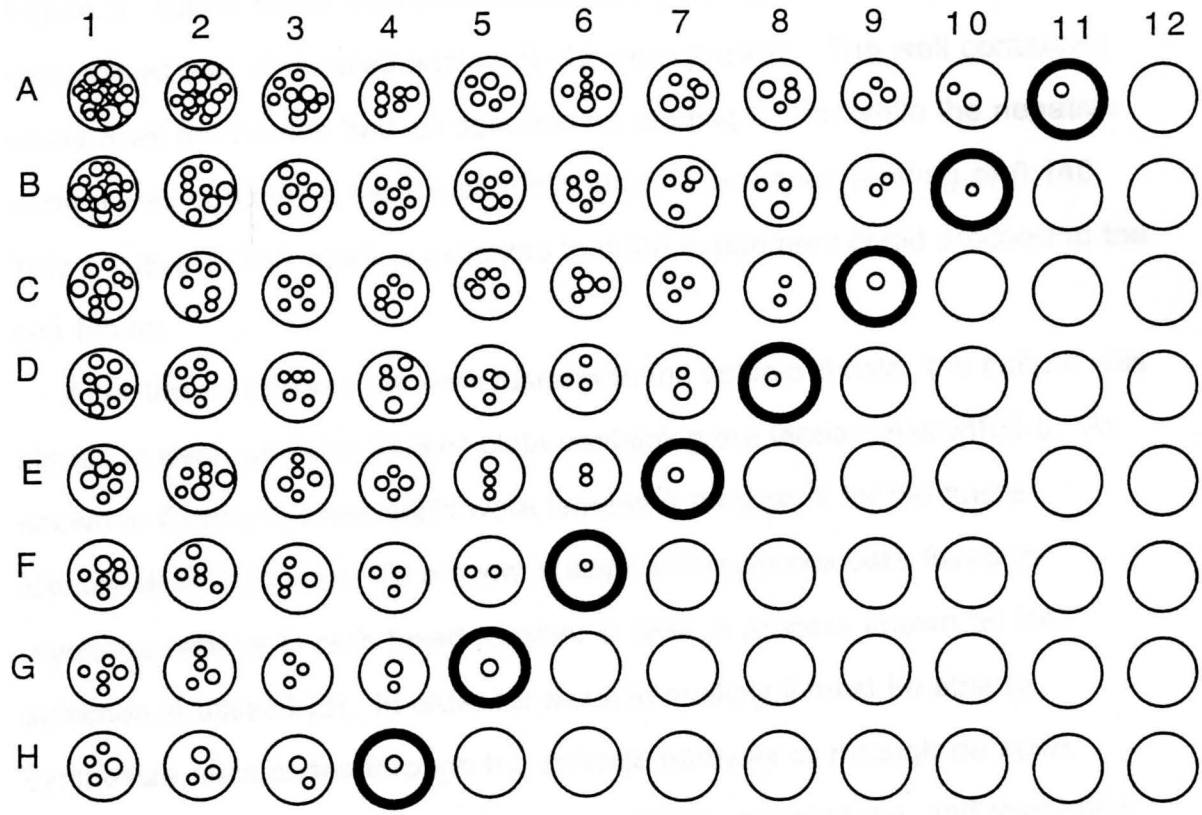
The fused cells were allowed to sit one week then observed under an inverted microscope and fed every two or three days with RPMI 1640 containing 20% heat inactivated fetal calf serum, penicillin/streptomycin, and L-glutamine. The

cells were kept at 37°C in a carbon dioxide incubator. When a hybridoma culture tested positive for antibody production, a sample was frozen and stored at -70°C. For cryopreservation, the cells were spun at 250 x g for ten minutes. The pellet was resuspended in 1 ml of complete RPMI 1640 containing 10% dimethylsulfoxide. Each aliquot was put in a cryogenic vial and stored in a taped Styrofoam box for 24 hours at -70°C. The vials were then transferred to their respective shelves in the -70°C freezer or to liquid nitrogen (8).

Limiting dilution cloning

A 96-well plate was obtained and seeded with MRC-5 cells to serve as a feeder layer. 50 ul of the MRC-5 cells at 1 x 10^5/ml were added to each well and grown to confluence. The media was removed and replaced with 50 ul complete RPMI 1640. Subcloning was performed by the rapid cloning method as described by Harlow and Lane in *Antibodies: A Laboratory Manual* (9). To the top left well 50 ul of hybridoma cells were added. The cells were mixed by gently pipetting and a 1:2 serial dilution was done down the left row. The remaining 50 ul was discarded. Using an eight well multipipettor, a 1:2 serial dilution was done across the plate. The cells were allowed to grow for 5-7 days and the plate was observed for wells containing only one cell. Theoretically a diagonal line from the top right corner to the bottom left corner should appear containing only one cell per well (see Figure 2). The wells containing only one cell were grown to confluence, transferred to a 24-well plate, and tested by ELISA. This procedure was repeated twice to produce a purified hybridoma culture.

Figure 2. Theoretical depiction of Limiting Dilution Cloning. A diagonal line of single cell wells extends from the bottom left to the top right.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RESULTS

The procedure that was followed throughout the project is summarized in Figure 3. When blood from the immunized mouse was tested by ELISA, it was determined that antibodies against IL-12 were present. The well containing blood from the mouse had an absorbance reading 0.675, while the negative control well containing mouse serum had an absorbance reading of 0.140. This positive ELISA reading indicated that the experiment could proceed to the cell fusion.

After the myeloma cells were fused with the splenic B cells, the culture was placed in each well of a 24-well plate containing the feeder layer MRC-5. As shown in Figure 4, these wells were labeled A through X for the future identification of clones. To prevent culturing of myeloma cells fused to myeloma cells or B cells fused to other B cells, a process known as HAT selection is utilized (8). In order for a cell to multiply it must be able to synthesize nucleotides through the salvage pathway or through *de novo* synthesis. HAT is an acronym for hypoxanthine, aminopterin, and thymidine. Aminopterin in the hybridoma culture media prevents *de novo* synthesis of nucleotides and forces all cells to utilize the salvage pathway. Hypoxanthine and thymidine both encourage use of the salvage pathway because they are intermediates. However, the only cells that can utilize the salvage pathway are those that are $HGPRT^+$. HGPRT stands for hypoxanthine guanine phosphoribosyl transferase and it is a vital enzyme of the salvage pathway. Myeloma cells are $HGPRT^-$ and B cells are $HGPRT^+$. Therefore a myeloma cell fused to another myeloma cell will not survive in culture because it cannot perform *de novo* synthesis due to the presence of aminopterin. It cannot utilize the salvage pathway because it is $HGPRT^-$. A splenic B cell fused to another splenic B cell wll synthesize nucleotides because it is $HGPRT^+$, but for an

Figure 3. Flow Chart of Procedure. This is a brief description of the methods used for the research project.

Fused B cells with myeloma cells and plated on a 24-well plate

↓

Tested surviving colonies for antibody production

↓

Limiting dilution cloning on positive clones

↓

Isolated and grew up single cell clones

↓

Tested the single cell clones for antibody production

↓

Further purification of single cell clones via limiting dilution cloning

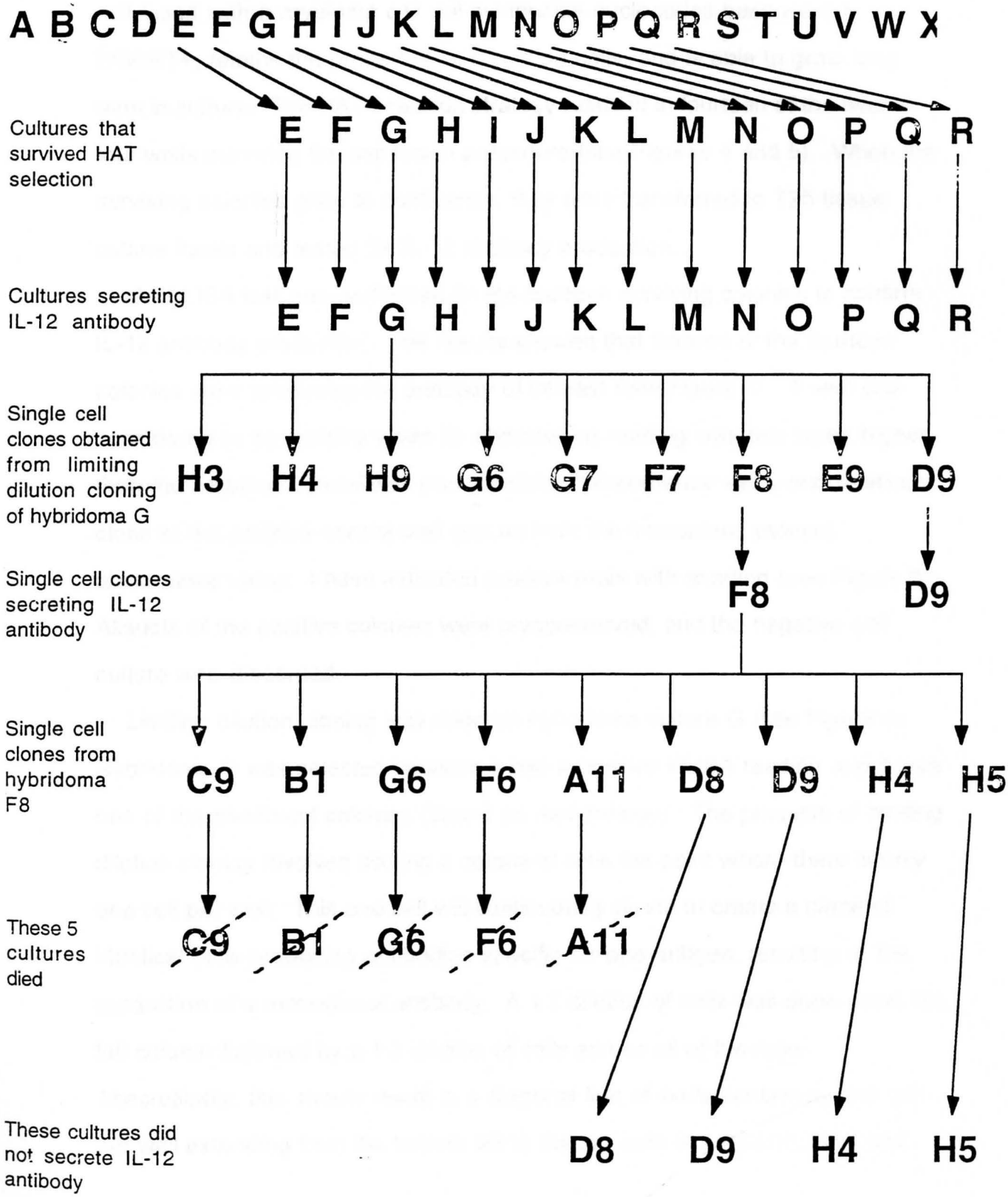
↓

Isolated and grew up the purified single cell clones

↓

Tested these clones for antibody production

Figure 4. Flow Chart of Subcloning Strategy and Results.


A B C D E F G H I J K L M N O P Q R S T U V W X
Cultures that survived HAT selection
E F G H I J K L M N O P Q R
Cultures secreting IL-12 antibody
E F G H I J K L M N O P Q R
Single cell clones obtained from limiting dilution cloning of hybridoma G
H3 H4 H9 G6 G7 F7 F8 E9 D9
Single cell clones secreting IL-12 antibody
F8 D9
Single cell clones from hybridoma F8
C9 B1 G6 F6 A11 D8 D9 H4 H5
These 5 cultures died
C9 B1 G6 F6 A11
These cultures did not secrete IL-12 antibody
D8 D9 H4 H5

unknown reason spleen cells show no long-term growth in culture. A splenic B cell fused with a myeloma cell will synthesize nucleotides because it is HGPRT+, retains the properties of myeloma cells, and is able to grow long-term in culture. The HAT selection strategy resulted in fourteen of the twenty-four wells surviving the cell fusion procedure (see Figures 4 and 5). When the surviving colonies grew to confluence, they were transferred to T25 tissue culture flasks and tested for IL-12 antibody production.

An ELISA test was performed on the fourteen surviving colonies to confirm IL-12 antibody production. The results showed that thirteen of the fourteen colonies were producing the antibody of interest (see Figure 4). A well was considered to be positive when its absorbance reading was two times higher than the negative control well (mouse serum) absorbance value and relatively close to the positive control well (serum from the immunized mouse) absorbance value. I have indicated positive wells with shading (see Figure 6). Aliquots of the positive colonies were cryopreserved, and the negative cell culture was discarded.

Limiting dilution cloning was done on hybridoma culture G (see Figure 4). Hybridoma G was selected because it had a positive ELISA reading and it was one of the healthiest colonies (based on morphology). The principle of limiting dilution cloning involves diluting a colony of cells the point where there is only one cell per well. This one cell will continuously divide to create a clone of identical cells producing antibodies specific for one antigen, resulting in the production of a monoclonal antibody. A 1:2 dilution of cells was done down the left column followed by a 1:2 dilution of cells across all of the rows. Theoretically, this should result in a diagonal line of wells containing one cell per well extending from the bottom left to the top right of a 96-well plate (see

Figure 5. 24-well plate of Surviving Hybridoma Colonies. The surviving cultures are encircled.

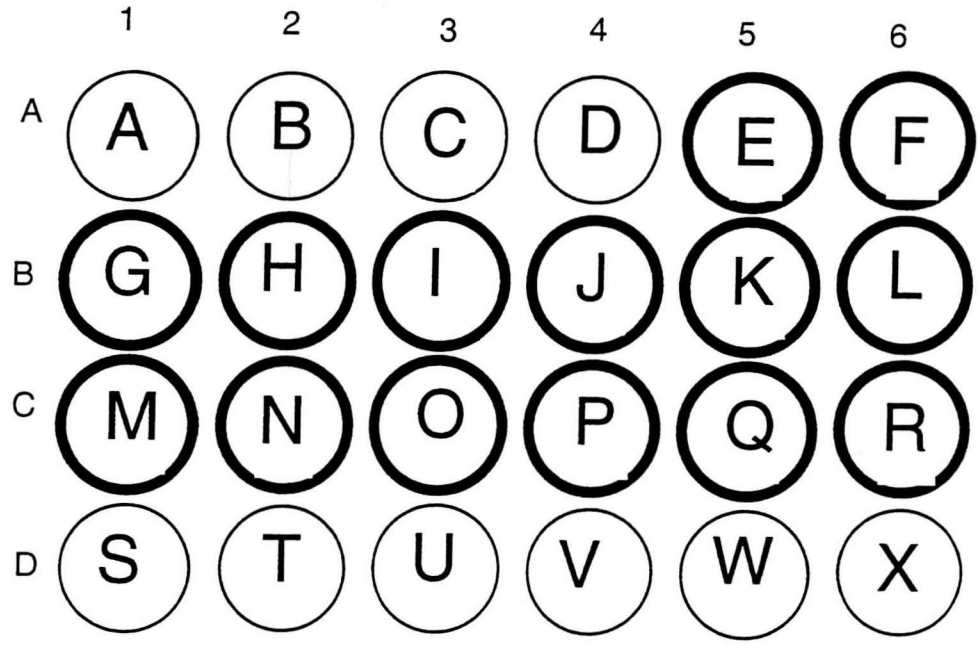

1 2 3 4 5 6
A A B C D E F
B G H I J K L
C M N O P Q R
D S T U V W X

Figure 6. ELISA of Surviving Hybridoma Colonies. A positive test result is indicated by shading. Well B2 is the positive control and B3 is the negative control. Cultures E, F, G, H, I, J, K, L, M, N, O, Q, and R are all positive. Absorbance values for each well: +control=*.***(too positive to be read), -control=2.504, E=2.752, F=1.576, G=0.919, H=1.420, I=2.77, J=1.037, K=0.907, L=1.072, M=1.808, N=1.171, O=1.408, P=0.249, Q=1.526, R=2.509.

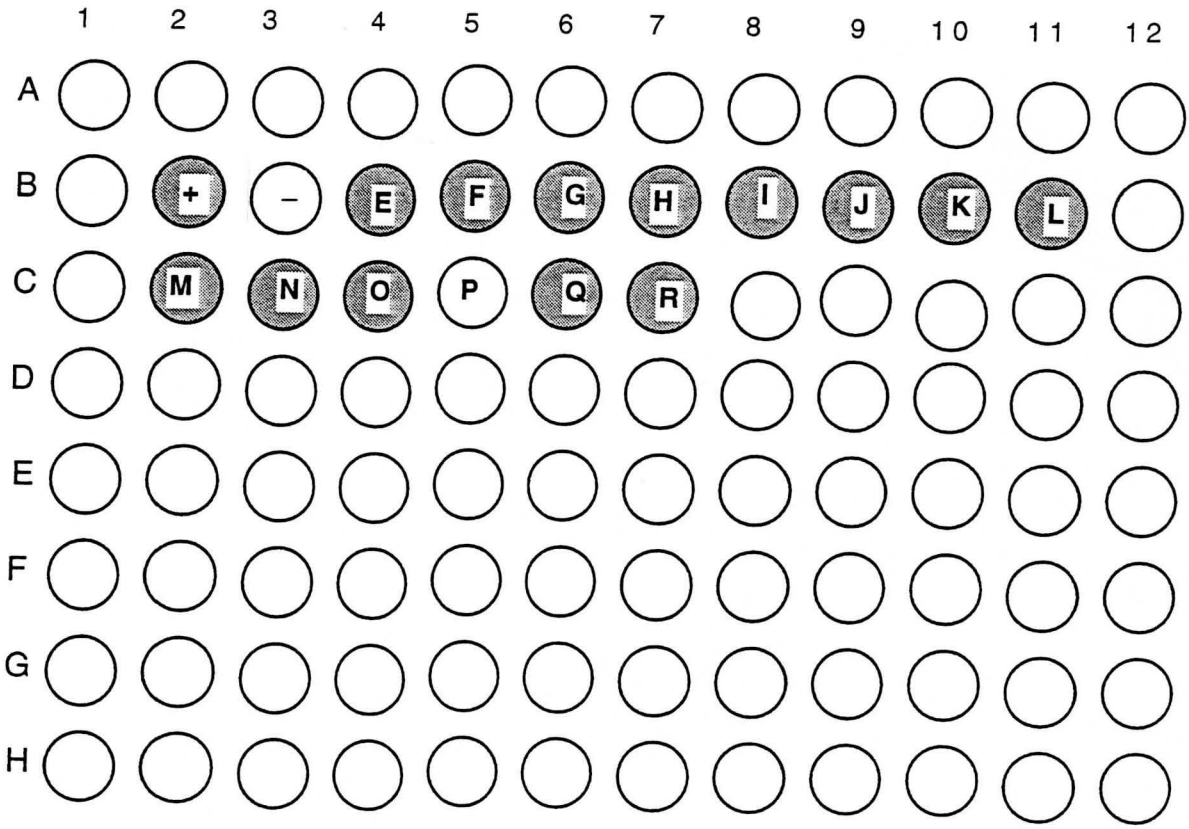

1 2 3 4 5 6 7 8 9 10 11 12
A
B + – E F G H I J K L
C M N O P Q R
D
E
F
G
H

Figure 7. First Purification by Limiting Dilution. Wells H3, H4, H9, G6, G7, F8, F7, E9, and D9 contain only one cell and are encircled.

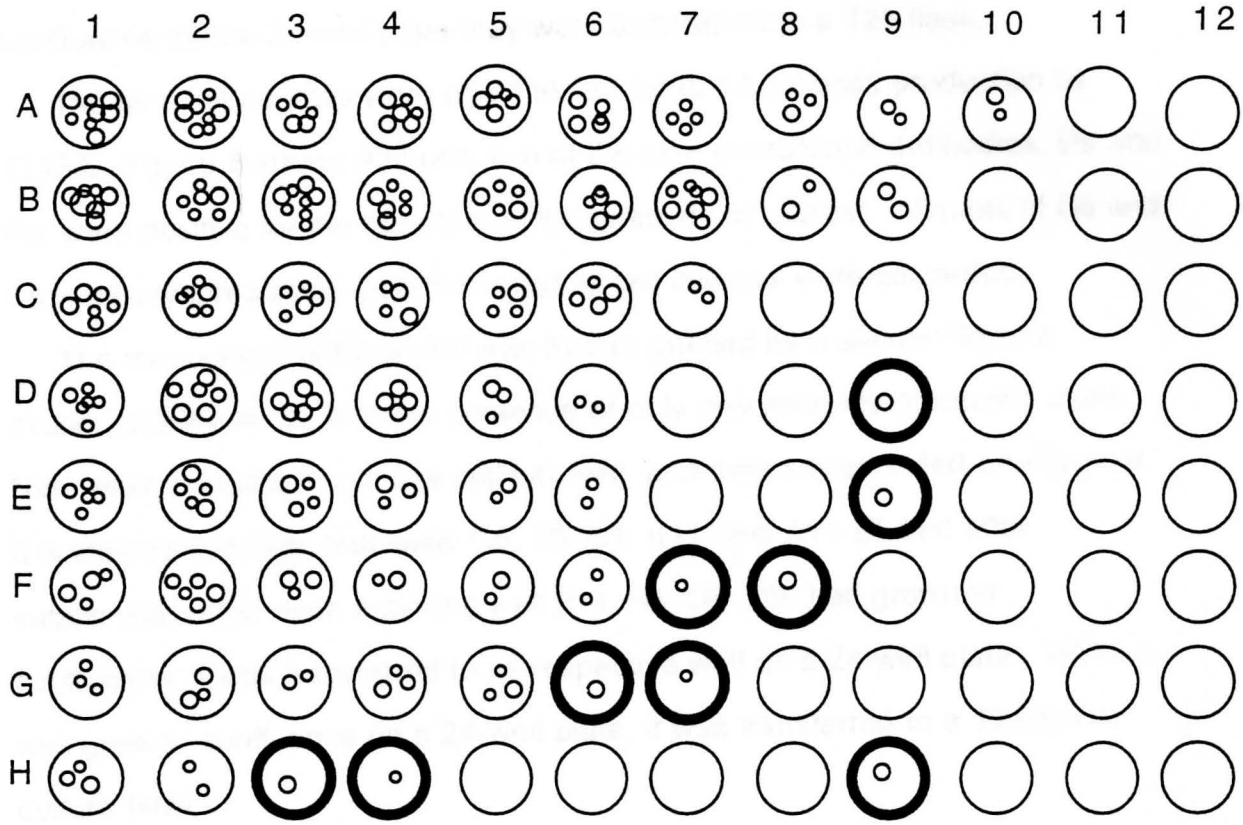

1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H

Figure 2) (8). Nine wells contained only one cell per well, and these are darkly encircled in Figure 7. Each of these nine wells were grown to confluence and transferred to their respective well on a 24-well plate. When the cells grew to confluence on the 24-well plate they were transferred to a T25 flask.

The nine hybridomas were again tested for IL-12 antibody production by ELISA. Figure 8 shows that only two of the nine monoclonal antibodies, D9 and F8, were positive for the secretion of the antibody of interest. Aliquots of D9 and F8 were cryopreserved, and the negative cell cultures were discarded.

The monoclonal antibody F8 was further purified by a second limiting dilution cloning to confirm the presence of only one antibody producing clone. Nine wells contained only one cell per well, and they are encircled on Figure 9. It is important to note that wells G6, F6, C9, B11, and A11 all died after subcloning. After each surviving well (H4, H5, D8, D9) had grown to confluence, it was transferred to its respective well on a 24-well plate. When a well grew to confluence on a 24-well plate, it was transferred to a T25 tissue culture flask.

The four surviving hybridomas were tested for IL-12 antibody production by ELISA (see Figure 10). The monoclonal antibody absorbance values were all close to the negative control absorbance value indicating the hybridomas were not secreting antibody directed against IL-12 (see Figure 11).

Figure 8. ELISA of Once Purified Single Cell Clones. A positive test result is indicated by shading. Well B2 is the positive control and B3 is the negative control. D9 and F8 are the only positive monoclonal antibodies. Absorbance values for each well: +control=1.819, -control=0.584, D9=1.973, E9=0.413, F7=0.492, F8=0.769, G6=0.583, G7=0.365, H3=0.340, H4=0.398, H9=0.389.

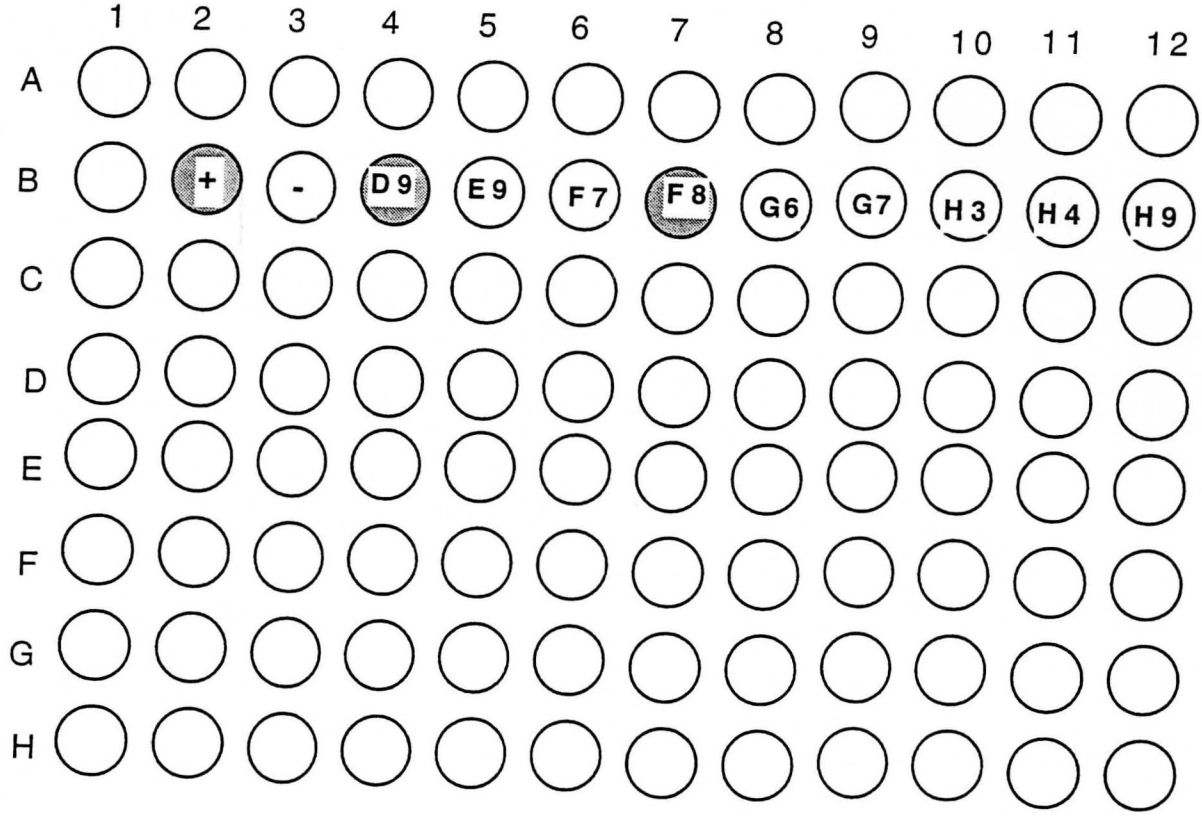

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
+
-
D9
E9
F7
F8
G6
G7
H3
H4
H9

Figure 9. Second Purification by Limiting Dilution. When monoclonal antibody F8 was purified by limiting dilution cloning, wells H4, H5, G6, F6, D8, D9, C9, B11, and A11 all contained only one cell. Hashed circles indicate cultures that died immediately after subcloning.

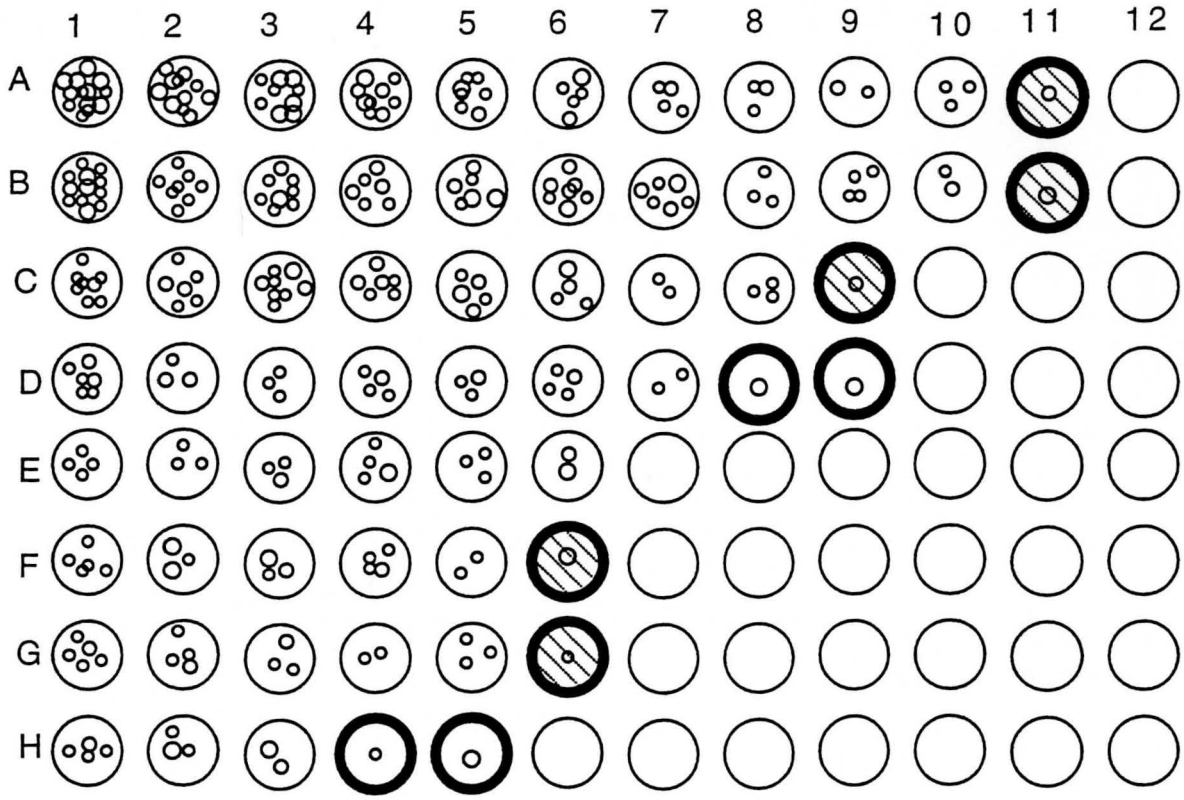

1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H

Figure 10. ELISA of Twice Purified Single Cell Clones. The ELISA was negative indicating no monoclonal antibody was secreting antibody directed against IL-12.

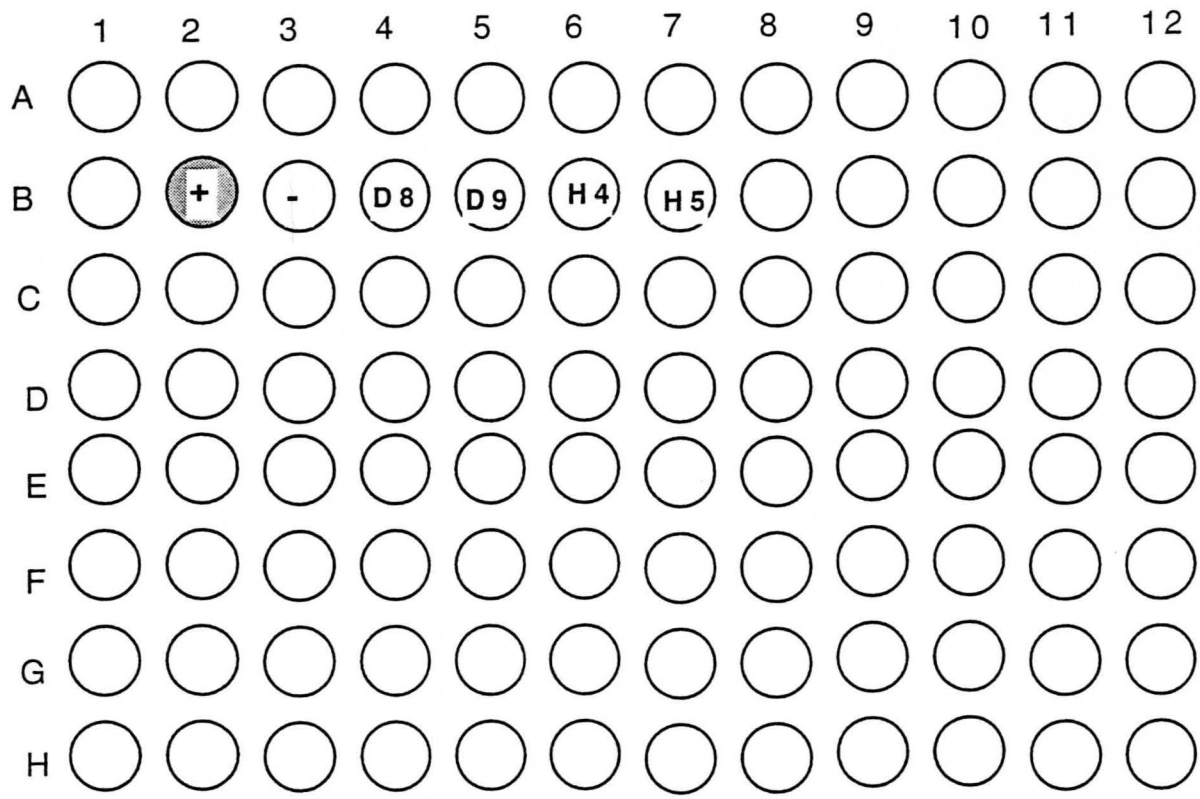
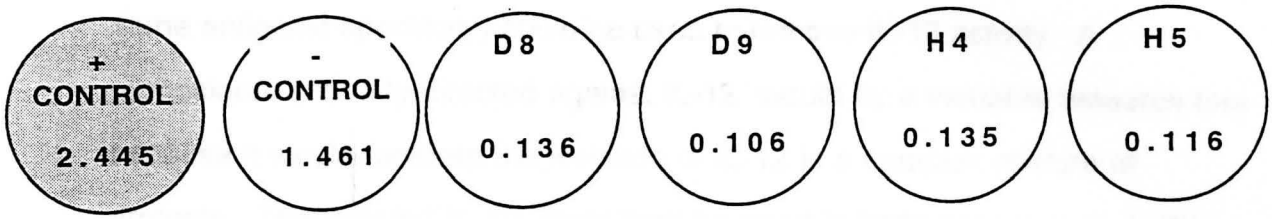

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
+
-
D8
D9
H4
H5

Figure 11. ELISA Absorbance Values of the Twice Purified Single Cell Clones. The absorbance values of the positive and negative control wells are shown in comparison to the four monoclonal antibodies, indicating that no monoclonal antibodies were positive.


+
CONTROL
2.445
-
CONTROL
1.461
D8
0.136
D9
0.106
H4
0.135
H5
0.116

DISCUSSION

In order to further understand the effect of IL-12 on NK cells, a monoclonal antibody (a homogenous preparation of antibody molecules all exhibiting the same antigenic specificity) could be used to remove IL-12 activity. A monoclonal antibody directed against IL-12 would by a valuable research tool because it would facilitate the isolation of IL-12 in a complex mixture of proteins. The isolated IL-12 could then be used in immunoassays to further study its effect on NK cells, and virally infected cells. After this project was initiated, it was discovered that IL-12 could be purchased commercially. Pure recombinant (made in bacteria) IL-12 can be bought commercially but IL-12 purified in this manner would be naturally synthesized and have the same activity as that seen in nature, while recombinant IL-12 may not. Additionally, antibodies to IL-12 may be used to eliminate IL-12 activity in animal or in a cell culture system. This allows the researcher to examine the effect of IL-12 in a natural system containing all of the other interacting cytokines, cells and growth factors.

I was able to successfully create a monoclonal antibody that was subcloned once. When an ELISA was done on the first subcloning of hybridoma cells, only two clones (F8 and D9) of the nine clones tested, were positive for the secretion of antibody directed against IL-12. When one of the positive subclones (F8) was subcloned a second time it lost its capability of secreting the antibody of interest. This is due to the unstable nature of hybridomas and their random chromosome loss. It is likely that the chromosome containing the gene responsible for IL-12 antibodies was no longer maintained in the cells. While subcloning one time may result in a pure population of cells (and thus, a monoclonal antibody), a second subcloning step ascertains the purity of the clone. In future studies, the positive clone D9 obtained from the limiting dilution

cloning of hybridoma G (see Figure 4) should be thawed and re-cultured. A second limiting dilution cloning should be done followed by confirmation of IL-12 antibody production by ELISA. If this procedure is ineffective in producing a twice subcloned monoclonal antibody that tests positive, an earlier hybridoma culture (prior to subcloning) can be re-cultured, subcloned by limiting dilution, and tested by ELISA.

After producing a positive monoclonal antibody directed against IL-12 the clone should be expanded. Expanding the clone involves growing a large amount of cells in T25 flasks. Aliquots of cells should be cryopreserved in the event of contamination and for later experimentation.

The isotype of the monoclonal antibody should be determined. This can be done by either Ouchterlony double-diffusion assay or antibody capture on antigen coated plates (7). The Ouchterlony double-diffusion assay involves a tenfold concentration of cell culture supernatant pipetted into a well in a bed of agar. Class and subclass-specific antisera are pipetted in other wells at equal distances from the test antibody. When the monoclonal antibodies and the antisera diffuse to meet, immune complexes will form if binding occurs. A line of proteins visible to the naked eye or visible by staining denotes the isotype of the monoclonal antibody (7). The second method for determining the isotype is similar to the previously performed ELISA. A 96-well plate is coated with IL-12 then cell culture supernatant is added to allow binding of the antibody to the antigen. A labeled anti-class specific immunoglobulin is then incubated in the wells to bind one particular class of immunoglobulin. If this class is the isotype of the monoclonal antibody a positive result will be obtained. This method should be repeated with different anti-class specific immunoglobulins to confirm the isotype (7).

Immunoglobulin isotypes are characterized by unique amino acid sequences in the heavy chain constant region resulting in different structures and effector functions (1). IgG is the most abundant isotype found in serum, and is comprised of four subclasses. IgG is a monomer consisting of two gamma heavy chains and two kappa or lambda light chains. The structural characteristics that distinguish the subclasses from one another are the size of the hinge region, and the number and position of the interchain disulfide bonds between the heavy chains. IgG_1, IgG_3, and IgG_4 protect the developing fetus. IgG_3 and IgG_1 are able to activate complement and bind with high affinity to Fc receptors on phagocytic cells. IgG_2 and IgG_4 are also able to bind these Fc receptors but with lower affinity (1).

Monomeric IgM is expressed as membrane bound antibody on B cells but secreted as a pentamer by plasma cells. In pentameric IgM the five monomer subunits are arranged with their Fc regions in the center of the pentamer and the ten antigen binding sites on the periphery of the molecule. Each monomer unit is linked by two disulfide bonds. Each pentamer contains an additional Fc-linked polypeptide known as the J chain. IgM is the first immunoglobulin class produced in a primary response to an antigen. The increased valency of pentameric IgM increases its capacity to bind multidimensional antigens such viral particles and red blood cells. Therefore fewer numbers of IgM pentamers are required to cause agglutination of red blood cells and neutralization of viral particles. IgM is also efficient at complement activation (1).

IgA is the predominant immunoglobulin class found in secretions such as breast milk, saliva, tears, and mucus of the bronchial, genito-urinary, and digestive tracts (1). Secretory IgA is found as dimers or tetramers with a J-chain polypeptide and a secretory component polypeptide. Secretory IgA exhibits effector functions at mucous membrane surfaces, the entry sites for

most pathogenic organisms. IgA can cross-link large antigens with multiple epitopes. It can bind bacterial and viral surface antigens and prevent their attachment and colonization in mucosal cells. Complexes of secretory IgA and antigen are easily entrapped in mucous and then eliminated by the ciliated epithelial cells of the respiratory tract or by peristalsis of the gut (1).

IgE antibodies mediate the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives, and anaphylactic shock. IgE binds Fc receptors on the membranes of blood basophils and tissue mast cells. Cross-linkage of receptor-bound IgE molecules by antigen induces degranulation of basophils and mast cells. This results in the release of mediators present in the granules that give rise to allergic manifestations. Localized mast cell degranulation induced by IgE also may release mediators that facilitate a buildup of various cells necessary for antiparasitic defense (1).

IgD together with IgM is the major membrane-bound immunoglobulin expressed by mature B cells. It is thought to function in the activation of a B cell by an antigen but its specific biologic function is not known. As IgD is not usually secreted by B cells, it is unlikely that it would be found in a hybridoma preparation (1).

After determining the isotype of the monoclonal antibody, it should be purified. There are several protocols for purification of mouse monoclonal antibodies. In a comparison conducted by Manil et al, monoclonal antibodies were purified by ammonium sulfate precipitation and/or gel filtration, anion exchange (DEAE), hydroxylapatite, and affinity (protein A) chromatography (13). The immunoglobulin yield was assessed along with the purity of the product by two-dimensional gel electrophoresis.

Salt precipitation and gel filtration consisted of the addition of a saturated ammonium sulfate solution to ascitic fluid, centrifugation, and resuspending

the pellet in distilled water. The filtered ascites was purified on a Sephacryl column and equilibrated with sodium phosphate buffer. This method was considered to be less effective in yield and purity than the other methods (13).

Another protocol, ion exchange chromatography (DEAE), involved ascitic fluid being dialyzed overnight with Tris-HCl buffer. The ascitic fluid was applied to a DEAE-Sephacel column and the elution was performed overnight with a NaCl linear gradient. DEAE chromatography provided a slightly better yield, but only moderate purity (13).

Hydroxylapatite chromatography involved ascitic fluid being applied to a column containing HTP DNA grade hydroxylapatite. The elution was performed with a linear or discontinuous NaCl gradient. A peristaltic pump was used to adjust the flow rate and collect the fractions. This method proved to be less efficient in terms of yield, purity, and day-to-day reproducibility (13).

Protein A chromatography consistently provided the preparation of immunoglobulin having the highest purity. It involved ammonium sulfate precipitation of ascitic fluid then passage over a protein A-Sepharose column. Protein A is a protein isolated from Staphylococcus Aureus bacteria that binds specifically to the Fc (functional) region of IgG, thus acting as an affinity ligand for immunoglobulin. The elution was performed using a discontinuous pH gradient with steps at pH 8.5, 5.8, 4.5, 3.5, and 2.2. The best results in terms of yield and purity were a function of the optimization of protein A concentration(13). A method was devised by Ey et al that allows the isolation of mouse IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins in almost 100% yield (14). Mouse serum or an immunoglobulin mixture is first passed over a protein A-Sepharose column. IgG1, IgG2a, and IgG2b will be eluted upon the addition of buffers at pH 6.0-7.0, pH 4.5-5.0, and pH 3.5-4.0, respectively (14).

Following purification of the antibody, the specificity of the monoclonal antibody should be determined. Cytokines similar in structure and function to IL-12 may bind to the monoclonal antibody, therefore tests comparing cytokine binding should be performed. It will also be important to determine which portion of the IL-12 heterodimer is bound by the monoclonal antibody. IL-12 is a 70kDa heterodimer composed of a 35kDa subunit and a 40kDa subunit. The monoclonal antibody may bind the p35 subunit, the p40 subunit, or the heterodimer (12).

The portion of the IL-12 molecule bound by the antibody is important because the biologic effects of the 40kDa subunit are different from the 70kDa heterodimer. The 40kDa subunit of IL-12 does not cause proliferation or enhancement of the cytolytic activity of natural killer cells (16). However, results obtained by site-specific chemical modification of a tryptophan residue at or near the active site of IL-12 suggest a direct role of the 40kDa subunit in interacting with the IL-12 receptor (20). Supernatant fluids from cultures of COS cells transfected with mouse p40 cDNA could inhibit several IL-12-dependent responses *in vitro*. This is due to p40 homodimers binding the IL-12 receptor, thus preventing the binding of IL-12 (16 and 19). Gillessen et al (16) demonstrated that the majority of the structural determinants required for binding of IL-12 to its receptor are contained within the p40 subunit, but p35 is required for signaling. Their studies also indicated that the p40 subunit interacts with the beta subunit of the IL-12 receptor. The alpha subunit of the IL-12 receptor is as of yet unidentified. Together, these studies suggest that the homodimeric p40 may be a suitable antagonist for studying the role of IL-12 in various immune responses *in vivo* as well as *in vitro* (16).

The human IL-12 p40 homodimer also antagonistically binds the IL-12 receptor and prevents mediation of biologic activity (17 and 18). The IL-12 p40

subunit contains the essential epitopes for receptor binding but a proper conformation required for high affinity binding is achieved only when p40 is associated with a p35 subunit or another p40 subunit (17).

When immunoassays are performed it will be important to know if the monoclonal antibody is able to bind free p40 or p35 subunits present in the system. Whether the antibody binds to p40, p35 or the heterodimer can be determined by ELISA, immunoprecipitation, immunodepletion, Western blot, and IL-12 receptor binding assay. An ELISA can be done to determine this (12 and 18). We would first have to separate p40 from p35 using reduction and gel chromatography. A 96-well plate is coated with the p40 subunit and the monoclonal antibody is then added. The secondary antibody [goat anti-mouse IgG+IgA+IgM (H+L) peroxidase labeled immunoglobulin] should then be added. Upon the addition of substrate there will be a color change if the monoclonal antibody was specific for the p40 subunit. No color change means the monoclonal antibody was not specific for p40, therefore the ELISA should be repeated by coating another 96-well plate with either the p35 subunit or the IL-12 heterodimer (12).

Immunoprecipitation of ^{125}I-labeled IL-12 involves attaching monoclonal antibodies from the cell culture supernatant to agarose beads. Labeled IL-12 is incubated with the beads to allow binding. The bound antibodies are released from the beads and the immunoprecipitated IL-12 is analyzed by SDS-PAGE to determine if one or both subunits was precipitated (18). Immunodepletion of IL-12 bioactivity is similar to the previous method except the IL-12 is not radioactively labeled. The antibody to be tested is bound to agarose beads. The beads are then incubated with an IL-12 solution and the beads are removed from the solution by centrifugation. The supernatant (IL-12 solution) is then tested for the ability to stimulate NK function. If the antibody in

question binds to active IL-12 (heterodimer), the supernatant will be depleted of IL-12 activity (18).

In Western blotting, an IL-12 preparation is separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The nitrocellulose membrane is probed with monoclonal antibody supernatant, then a secondary antibody conjugated with an enzyme. Upon the addition of substrate, the presence of immune complexes can be determined by the development of colored spots of the filter paper. The position of the spots, relative to molecular weight markers will indicate the size of the molecule bound by the antibody (18).

Comparative studies between the monoclonal antibody and a commercially available monoclonal antibody directed against IL-12 should be done. R&D Systems markets a monoclonal antibody specific for heterodimeric IL-12 with no reactivity with free p40 (5). The Department of Inflammation/Autoimmune Disease at the Roche Research Center developed the monoclonal antibody directed against IL-12 (12).

In summary, the fusion of myeloma cells with antibody producing B cells resulted in the formation of a hybridoma culture that secreted antibodies directed against IL-12. The hybridoma culture was subcloned twice and subsequently lost the ability to secrete IL-12 antibodies. The continuation of this research project should include the re-culture of the hybridomas followed by subcloning to create a monoclonal antibody maintaining positive antibody secretion. Purification should be done after the monoclonal antibody is created. Isotypying studies will determine the class and subclass the monoclonal antibody. Specificity studies will determine the portion of IL-12 that is recognized and this will enable bioassays for the removal of cytokine activity to be conducted. These bioassays will provide information for the possible use of IL-12 in the treatment of cancer and virally infected cells.

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VITA

I was born in Youngstown, Ohio to Robert D. Beal and the late Marjorie L. Beal (1944-1988) on March 23, 1971. I attended Immaculate Conception School and graduated from Ursuline High School in 1989. I matriculated at Fisk University in Nashville, Tennessee and graduated in May of 1993 with a Bachelor of Arts degree in Biology. I worked three years as a Histotechnician at Corning Clinical Laboratories in Youngstown, Ohio. I began my graduate studies at Youngstown State University in March of 1994. My permanent address is: 836 Alameda Avenue, Youngstown, Ohio, 44510.