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GENERATION OF A HUMAN AND/OR MURINE MONOCLONAL ANTIBODY AGAINST HUMAN MAMMARY CARCINOMA

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GENERATION OF A HUMAN AND/OR MURINE MONOCLONAL ANTIBODY AGAINST HUMAN MAMMARY CARCINOMA

By

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ABSTRACT

GENERATION OF A HUMAN AND/OR MURINE MONOCLONAL ANTIBODY AGAINST HUMAN MAMMARY CARCINOMA

By

John Adam Gerlach

An attempt to generate a monoclonal immunoglobulin directed against human mammary carcinoma was made. The evidence supporting the generation of such an immunoglobulin is that hosts develop immune responses to antigens expressed by tumors. By using axillary lymph nodes from humans with mammary carcinoma as the source of lymphocytes for fusion, the immunoglobulin produced would be of human origin. Since human x murine fusions are unstable, mice were immunized with tumor material and used to generate a murine immunoglobulin directed against human mammary carcinoma. Immunofluorescent and avidin-biotin immunoperoxidase methods were used for screening of supernatants and immunized mouse sera. Neither a human nor a murine monoclonal antibody was generated at the termination of this project. Possible reasons for this were the instability of human x murine fusions and the fact that only two tumors with corresponding lymph nodes were used.

ACKNOWLEDGEMENTS

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INTRODUCTION

Tumors are known to express antigens that are considered to be "foreign" or "non-self" by the host (1,2,3,4,5). These antigens may be divided into three categories: embryonic, viral related and tumor specific (2,5). It is thought, though unproven, that carcinoma of the breast may be a tumor which is viral induced (2). Tumors of viral origin may express a portion of the viral genome on the membranes of tumor cells, thus producing the "foreign" or "non-self antigens.

It has been demonstrated that the host immune system responds to these tumor antigens in the form of cellular and humoral immune responses (1,2,4). This concept of "immune response to tumors" is supported by the evidence that animals who have had a tumor excised and the same tumor re-introduced will remain tumor free (2,5). Further support is offered by the observation that resistance to a tumor induced in one animal can be transferred to a different animal and enable it to reject a tumor with the same markers (2,5). Stimulation of an immune response by specified tumor antigens results in a decreased incidence of tumors when oncogenic viruses are injected. A good examine of this is seen in the effectiveness of Feline Leukemia Virus Vaccine (2,4,5). If these concepts are applied to a human model, people with mammary carcinoma should have an anti-tumor antibody.

There is evidence that these concepts can be applied to human models. Histologically, a tumor lesion classically has inflammatory cells (including plasma cells) associated with it. The lymph nodes proximal to

this tumor characteristically show the same involvement (2,5). Lymph nodes proximal to a tumor lesion are exposed to products of that tumor due to the drainage of lymph from that area. This lymph will possess the tumor antigens expressed by that particular tumor due to secretion of the antigen if it is a hormone or enzyme or by shedding of the surface antigens due to membrane blebbing or cell death. If an immune response is present, the B-lymphocytes in the proximal lymph node will be stimulated by these antigens.

In the mid-1970's, Köhler and Milstein pioneered the process of hybridoma production providing unlimited supplies of monoclonal antibodies (6,7). This process involves the fusion of B-lymphocytes with myeloma cells resulting in the "hybrid" of the two cells referred to as a hybridoma. The resultant hybridomas secrete the antibody of choice in culture as long as they are maintained. This original work was a murine x murine fusion.

Some investigators have had success in performing human x murine fusions (7,8,9,10). Several of these investigators have applied the concepts of immune response to produce monoclonal antibodies against tumor-associated antigens (9,10). Other investigators have also used a murine x murine fusion to produce anti-tumor monoclonal antibodies (7). This has involved immunization of the mouse with the tumor cells or tissue homogenate prior to fusion to elicit the desired immune response. The spleen of the animal was the source of the B-lymphocytes for fusion.

The production of a monoclonal antibody to a human mammary carcinoma could have possible diagnostic or therapeutic applications (7,11,12,13,14, 15,16). A highly specific antibody could be used for screening for early detection of mammary carcinoma, as an imaging tag for tumor definition or

to locate possible metastases in radiologic diagnostic work if appropriately tagged. Much investigation is going on to see if Ehrlich's "Magic Bullet" concept is realistic. This involves the delivery of anti-tumor agents linked to anti-tumor antibody directly and only to the tumor mass.

This project's premise was to use the concept of immune response to tumor antigens and the monoclonal technology available to produce a hybridoma that secretes an anti-mammary carcinoma. The use of human axillary lymph nodes proximal to a tumor lesion would result in the production of an antibody of human origin. Lymphocytes from this node were fused with murine myeloma cells. Because of the instability of human x murine fusions, mice were also immunized with tumor material and murine x murine fusions performed (7). Hybridoma supernatants and mouse sera were screened for immunoglobulin production against the immunizing tumor using indirect immunofluorescent and immunoperoxidase assays. These techniques were chosen over other methods for their sensitivity and safety over other methods (17,18,19,20,21,22,23,24).

MATERIALS

Tissue Material:

Portions of two different tumors with corresponding lymph nodes were used for this project. The first tumor (C.C.) received was described as a massive poorly differentiated infiltrative ductal carcinoma of the breast with extensive invasion of the axillary nodes. The second tumor (E.G.) was described as a well differentiated carcinoma of the breast without axillary node invasion.

Tumor Cell Lines:

Two myeloma cell lines were used in this project. The line used for fusion was CRL-1580. It is non-secreting and produces stable fusions. The other line that was used, CCL-130(MOPC-31C), secretes IgG. Both of these cell lines were obtained from the Americal Type Culture Collection.

METHODS

Tumor Cell Line Maintenance:

The CRL-1580 myeloma cells were cultured in a humidified 5% CO_2 environment at 37C. They were split one-to-ten every two to three days for maintenance of the cell line using Bull's Medium. In preparation for fusion, the cells were split one-to-two twice to have the cells in log phase growth.

The CCL-130 myeloma cells were cultured using CCL-130 Medium in a humidified 5% CO_2 37C environment. They were split one-to-four every two to three days to maintain the cell line. Some flasks of cells were allowed to grow until the media turned acid. The supernatant was then collected and frozen for future use.

Human X Murine Fusion:

Lymphocytes from the draining regional lymph node were obtained by perfusion of the node with Dulbecco's medium with 20% fetal calf serum using a 25 gauge needle. The cells collected were then pelleted and the supernatant was discarded. The pellet was resuspended with a NH_4C_1-EDTA solution to lyse any red blood cells present. The cells were then washed three times with the Dulbecco's fetal calf serum medium. The rest of the lymph node was ground with a tissue homogenator. The resultant homogenate was processed in the same manner and used for subsequent fusions.

The suspension of human mixed lymphocytes and a suspension of CRL-1580 cells were then washed separately twice with Dulbecco's without fetal calf serum. A manual cell count on each suspension was performed using a hemocytometer. A mixture of human mixed lymphocytes and CRL-1580 myeloma cells was made using a ratio of 10:1 respectively. This mixture was then washed once more using Dulbecco's without fetal calf serum. To the resultant pellet, 0.2 ml of 30% polyethylene glycol (MW 1000) was added and incubated at 37C for two minutes. This was then centrifuged for ten minutes at 1000 g. Once pelleted, 5 ml of serum free Dulbecco's was added while gently agitating the mixture. This was followed by the addition of 5 ml of Dulbecco's with 20% fetal calf serum. The suspension was then centrifuged and washed twice with Dulbecco's with 20% fetal calf

serum. Resuspension of the pellet after the final wash with 24 ml of hybridoma (HY) medium preceded the aliquoting of 1 ml fractions into a 24 well tissue culture tray. Hybridoma medium contains 62% Dulbecco's, 20% fetal calf serum, 10% NCTC 109, 1% non-essential amino acids, 1% HEPES buffer, 1% sodium pyruvate, 1% bovine insulin, 1% thymidine, 1% oxaloacetic acid, 1% hypoxanthine, 1% glutamine and 1% penicillin and streptomycin. All cultures were grown in a humidified 37C incubator with 5% CO_2.

The next day, each well was fed 0.5 ml HY medium with 0.4 uM aminopterin (1XHAT). On the second day, each well was fed 0.5 ml HY medium with 0.8 uM aminopterin (2XHAT). Cultures were then fed 0.5 ml HY medium weekly. Cultures were periodically examined microscopically using an inverted phase microscope to determine if there were hybridoma formation.

Supernatants of the cultures were collected at 21 and 35 days post-fusion to be used in immunologic assay techinques to determine if there were production of anti-tumor antibodies.

Indirect Immunofluorescent Screening:

An immunofluorescent technique was done using 4μ frozen sections of the appropriate tumor as a source of antigen. The sections were air dried for ten minutes and then oven dried for one hour. Sections were rinsed for ten minutes in phosphate buffered saline (PBS), overlaid with hybridoma supernatants and incubated at 37C for one-half hour. Three 15-minute rinses with PBS were performed prior to overlaying the sections with goat anti-human IgG fluorescein tagged antibody. This was incubated for one-half hour and followed with three 15-minute rinses with PBS. Sections

were then air dried and coverslipped using a 90% glycerol 10% PBS mounting medium. The sections were then examined under a fluorescent microscope to determine if any of the supernatants had anti-tumor activity.

Avidin-Biotin-Peroxidase Staining:

An avidin-biotin-peroxidase technique was used (Vectastain ABC kit). Four micron frozen sections of the appropriate tumor were used as a source of antigen. This technique used ten minute rinses between steps and incubation periods of 20 minutes with each reagent. An initial blocking of endogenous peroxidase activity was done using a 0.3% hydrogen peroxide (H_2O_2) in methanol solution. Blocking of endogenous binding of other components in the system was done by incubation with a 0.01% avidin solution followed by a 0.01% biotin solution. A final blocking of endogenous goat globulin binding was performed using a 2% normal goat serum in PBS solution. The culture supernatants were then overlaid followed by a biotinylated goat anti-human IgG antibody. A solution of avidin-biotin-peroxidase complex was then overlaid. A chromogenic substrate consisting of equal volumes of 1 mg/ml diaminobenzidine in TRIS/HCl buffer and 0.03% H_2O_2 in distilled water was used. The sections were then counterstained with Ehrlich's Hematoxylin and coverslipped with a permanent mounting media. The presence of a microscopically visible brown/black deposit indicated a "positive" or reactive staining pattern.

Enzyme-Linked Immunosorbant Assay(ELISA) Screen for Human IgG Production:

An enzyme-linked immunosorbant assay was also used to determine if the cultures were producing any immunoglobulin (IgG). This was a

modification of the above avidin-biotin-immunoperoxidase staining procedure. To facilitate the binding of any IgG antibodies in the culture supernatants to the polyvinyl chloride (PVC) microtiter plates, they were incubated for 4 hours in an alkaline pH buffer (50 mM bicarbonate buffer, pH 9.6) solution. Any "non-specific" binding sites on the plastic wells were blocked by the use of 0.1% crystalline bovine serum albumin solution. A further block using 2% normal goat serum was used. Incubation of the biotinylated goat anti-human igG was followed by the addition of the avidin-biotin-peroxidase complex. The chromogenic substrate used in this assay consisted of equal volumes of 0.8 mg/ml o-phenylenediamine dihydrochloride in 100 mM citrate/phosphate buffer solution and 0.03% H_2O_2 in distilled water solution. The color was allowed to develop and the absorbances for each well were obtained using a BIOTEK microtiter plate reader.

Immunization of Balb/C Mice with Tumor Material:

Pre-immune serum was obtained by bleeding the mice prior to injection of tumor material. Blood was collected by anesthetizing the mice with ether and then using capillary tubes to rupture and collect the blood from the median occipital vein. The serum was collected after centrifugation and then heat inactivated for 30 minutes at 56C. This was stored at -20C until used.

These same Balb/C mice were then injected intraperitoneally with either free tumor cells (10,000 cells/injection) or a homogenate of tumor tissue (250 µg of homogenate/injection) every 2 days for a total of 3 injections. Seven days after the last injection post-immune serum was collected and processed in the same manner as the pre-immune serum.

Screening of Mouse Serum Using Avidin-Biotin Peroxidase Staining:

Dilutions of 1:10, 1:20 and 1:40 in PBS of both pre- and post-immune mouse sera were used to avoid the inherent "false positive" non-specific binding of mouse immunoglobulins. The paired dilutions of pre- and post-immune mouse sera were used with the avidin-biotin-peroxidase method described earlier with modifications. Four micron frozen sections of tumor were used as the antigen source. A 2% normal horse serum to block endogenous binding of horse globulins and a biotinylated horse anti-mouse IgG were used. Mice whose post-immune serum gave a positive staining pattern at a higher dilution than their pre-immune serum were considered to have mounted an immune response to the tumor with which they were injected.

Murine X Murine Fusions:

Mice that were considered to have an immune response were sacrificed by dislocation of their cervical vertebrae. Their spleens were removed by dissection and ground with a tissue homogenator to remove the lymphocytes needed for fusion. The homogenate obtained was washed with NH_4C_1 to remove red blood cells and processed in the same manner as the mixed lymphocytes in the human x murine fusion technique described earlier. The myeloma cell line used for fusion was CRL-1580.

The fusion protocol used was the same as that used in the human x mouse fusions. The only deviation was in the use of polyethylene glycol (MW4000) for a portion of the mixed lymphocyte-myeloma mixture in an attempt to increase hybridoma production. This portion of the pellet had the MW 4000 polyethylene glycol added and was allowed to

incubate at room temperature for five minutes and then slowly diluted with 50 ml of Dulbecco's medium while gently agitating. From this point, the fusion was carried out in the same manner for both portions of the mixed lymphocyte-myeloma suspensions.

Feeding of 1XHAT, 2XHAT and weekly HY feedings were the same as in the human x murine fusion. All cultures were incubated at 37C with 5% CO_2 and examined microscopically for hybridoma formation weekly. Supernatants were harvested at 3 and 5 weeks post-fusion for screening.

ELISA Screen for Murine Anti-Tumor Antibody Production:

Microtiter trays were prepared with antigen by plating the antigen material, free cells (1000/well) or tissue homogenate (10 µg/well), in a 50 mM bicarbonate solution, pH 9.6, and incubated at 4C for 16-24 hours. Trays were prepared with tumor material (cells or homogenate) and normal breast material (cells or homogenate). This was done in order to differentiate non-specific antibody attachment from anti-tumor antibody attachment. Supernatants were run in parallel on trays with tumor material and on trays with normal breast material. Delta Absorbances (ΔABS) between the paired trays were used to calculate a mean ($\bar{x}$) and standard deviation (SD). A supernatant that gave a ΔABS greater than the $\bar{x}$ + 2SD was considered significant and further screening would be performed.

The avidin-biotin-peroxidase procedure used with the culture supernatants was similar to the avidin-biotin-peroxidase enzyme-linked immunosorbant assay used for screening cultures for human IgG production described earlier. Because the antigen source was tissue homogenate or free cells, all wells were blocked with 0.3% H_2O_2, avidin, biotin and

normal horse serum solutions prior to the addition of culture supernatants. Biotinylated horse anti-mouse IgG was used as a secondary antibody and preceded the addition of the avidin-biotin-peroxidase complex. The chromogenic substrate was the o-phenylenediamine dihydrochloride/H_2O_2 mixture described earlier. Once the color had developed, the supernatants were transferred to clean PVC trays.

Any supernatant giving a ΔABS value greater than $\bar{x}$+2SD would be tested by an assay similar to the avidin-biotin-peroxidase screen used on pre- and post-immune mouse sera. The culture supernatant would be run in parallel with the supernatant of CCL-130 (American Type Culture Collection) mouse myeloma cells. CCL-130 cells secrete immunoglobulin. The protein concentration of the CCL-130 supernatant would be adjusted to match that of the culture supernatant and run on 4μ frozen sections of the tumor using the avidin-biotin-peroxidase protocol. If the culture supernatant had a positive staining pattern and the CCL-130 did not, then the hybridoma was considered to be secreting an anti-tumor antibody and would be sent to Butterworth Hospital in Grand Rapids, Michigan for further evaluation of its clinical usefulness. If both the culture and the CCL-130 supernatants gave positive staining patterns, the hybridoma was considered to produce a non-specific antibody and discarded. The experimental protocol called for screening a library of soft tissue tumor sections using the supernatants of value by the avidin-biotin-peroxidase method.

Freezing of Hybridomas;

A semi-automated freezing system using liquid nitrogen was used. The cells to be frozen were washed free of culture media and

resuspended in freezing medium at a density of 1,000,000 cells/ml. The freezing medium was kept at 4C prior to the resuspension. One milliliter aliquots of the suspensions were placed into tubes and kept in an ice bath until placed in the 4-5C precooled chamber. Once the tubes were in the sealed chamber, the instrument was placed in automatic mode and cooled in stepwise intervals until the desired temperature was reached. At this point, the tubes (with the cells) were removed and stored in liquid nitrogen until needed.

Thawing of Frozen Cells:

Cells to be thawed were removed from the liquid nitrogen storage tank and immediately placed in a 37C water bath. As soon as the 1 ml aliquot within the tube was thawed, it was transferred to a tube with 9 ml of the medium in which the cells were grown. This mixture was then centrifuged and the pellet resuspended with fresh medium. The suspension was then placed into culture in a humidified 37C incubator with 5% CO_2.

RESULTS

Human x Murine Fusion:

Three human x murine fusions were performed. Two of these fusions used lymphocytes obtained by perfusion of lymph nodes from patients identified as C.C. and E.G. The third fusion used lymphocytes obtained from the homogenate of the C.C. lymph node.

Of the three human x murine fusions performed, only one hybridoma was produced. It was seen on the thirteenth day following fusion of C.C. lymphocytes obtained by perfusion. The hybridoma was a cluster of sixteen well-rounded cells with defined margins. On the fourteenth day following fusion, this hybridoma was no longer visible. The supernatants from this fusion were screened using the indirect immunofluorescent technique. Distinguishing a reactive fluorescent pattern from a non-reactive pattern using this technique was very difficult due to the amount of autofluorescence produced by the tumor tissue itself. The immunofluorescent technique was not used for future screening of culture supernatants because of its inherent problem. Screening of these and the subsequent fusion culture supernatants using the avidin-biotin-peroxidase method on 4μ sections failed to yield any supernatants giving a reactive staining pattern.

The only fusion that was screened for human IgG production was the third and final human x murine fusion that used the lymphocytes obtained from the homogenate of the C.C. lymph node. A mean absorbance of 0.071 with a standard deviation of 0.029 absorbance units was obtained. None of the supernatants screened had an absorbance greater than the 0.129 absorbance units needed to indicate significant IgG production ($\bar{x}$+2S.D.).

Murine x Murine Fusions:

Two sets of six mice were used for immunization. Three mice of each set were injected with tumor material from C.C.; the other three received tumor material from E.G. One mouse injected with C.C. tumor material and one mouse injected with E.G. tumor material from each set were chosen to be fused. A total of four murine x murine fusions were accomplished.

Numerous hybridomas were visible microscopically 12-14 days post-fusion in all four of the fusions performed. The supernatants of the two C.C. fusions had ΔABS means of 0.001 and 0.002. Their respective standard deviations were 0.042 and 0.022. None of the individual supernatants tested had a ΔABS greater than $\bar{x}$+2SD and all were therefore considered insignificant. The supernatants of the two E.G. fusions were also tested using the ELISA technique and had ΔABS means of 0.063 and 0.122. Their respective standard deviations were 0.045 and 0.036. None of these supernatants had an individual ΔABS value that was greater than $\bar{x}$+2SD. These fusions did not produce an antibody directed against human mammary carcinoma.

The experimental protocol of this project called for the avidin-biotin-immunoperoxidase screening of supernatants of value in parallel with the CCL-130 supernatant. This portion of the protocol was never actualized. No supernatants were sent to Butterworth Hospital in Grand Rapids, Michigan for evaluation of its potential utility.

DISCUSSION

Much of the time and effort afforded this project involved perfection of the screening methods. During this project, it was found that undiluted mouse sera overlaid on tissue sections bind non-specifically leading to "false" positive results (see figures 1 and 2). Parallel serial dilutions of pre- and post-immune serum can overcome this problem (see figures 3 and 4). It was also found that breast tissue has endogenous avidin binding activity (24) and must be blocked with avidin and biotin solutions to make it possible to use an avidin-biotin-peroxidase staining procedure. While the production of a monoclonal antibody directed against human mammary carcinoma was not actualized, the screening methods established can be used for future projects of similar intent.

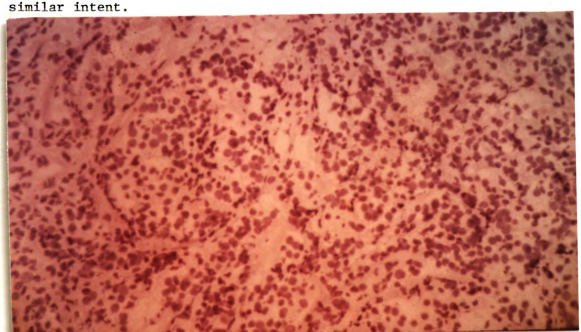

Figure 1. Avidin-biotin immunoperoxidase negative control. This section of tumor tissue was stained using the avidin-biotin immunoperoxidase staining methodology. The addition of mouse serum was omitted and replaced with phosphate buffered saline at that step in the procedure.

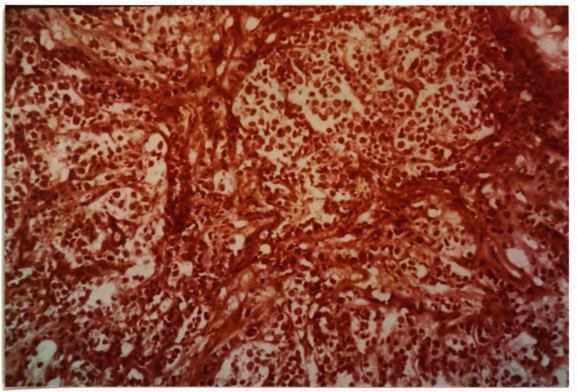

Figure 2. Avidin-biotin immunoperoxidase staining using normal mouse serum. This section of tumor tissue was stained using the avidin-biotin immunoperoxidase staining methodology. Undiluted serum from a mouse that was not immunized with tumor tissue was used as the source of primary antibody. It was not expected to show deposition of a brown/black pigment.

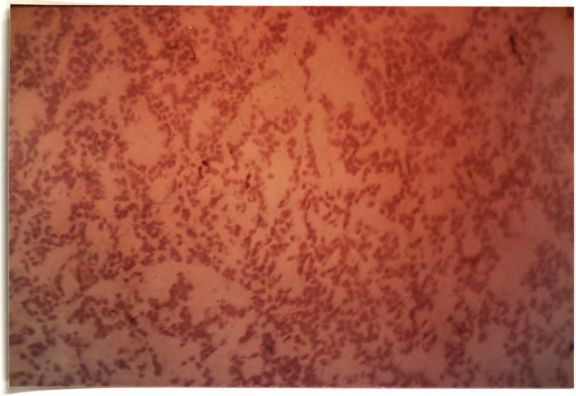

Figure 3. Avidin-biotin immunoperoxidase staining using a 1:20 dilution of pre-immune mouse serum. This section of tissue was stained using the avidin-biotin immunoperoxidase staining methodology. Serum from a mouse not immunized with tumor tissue was used as the source of primary antibody. This serum was diluted 1:20 in phosphate buffered saline. There is no deposition of a brown/black pigment indicating a non-reactive result.

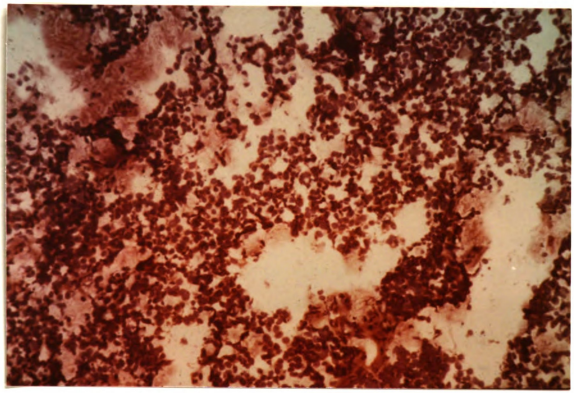

Figure 4. Avidin-biotin immunoperoxidase staining using a 1:40 dilution of immune mouse serum. This section of tissue was stained using the avidin-biotin immunoperoxidase staining methodology. Serum from the same mouse as in figure 3 was collected after immunization with tumor material and diluted 1:40 in phosphate buffered saline and used as the source of primary antibody. The deposition of a brown/black pigment indicates a reactive result.

RECOMMENDATIONS

Given the opportunities of more time and more tumors with corresponding lymph nodes, the following modifications of the procedures used in this project may yield the monoclonal immunoglobulin sought. Procurement of a non-secreting human myeloma cell line would be the most significant change that could be made. The use of such a cell line would enable an investigator to generate stable hybridomas using human lymphocytes. Unfortunately, such a cell line is currently unavailable. Fusion with rat myeloma cells (CRL-1631) may offer more numerous and more stable fusion partners for human B-lymphocytes. Also, the use of heteromyelomas (25) may also offer the same advantages over murine cell lines. Heteromyelomas are the product of the fusion of mouse myeloma cells and non-secreting human lymphocytes. It is thought that human chromosomes would less likely be lost using these as fusion partners and thus produce stable hybridomas. This variation, coupled with the recent advances at Johns Hopkins University (26) using avidin labelled antigen to select only the specific B-lymphocytes for electro-fusion with biotinylated myeloma cells, may offer the key to the success of this project. Only time, specimens and further investigation can answer these questions.

APPENDIX

Appendix 1

Reagents:

A. Media

1). Bull Media	83 ml	Dulbecco's with $NaHCO_3$
	1 ml	HEPES Buffer
	1 ml	Non-essential Amino Acids
	10 ml	Fetal Calf Serum
	0.45 ml	Na Pyruvate
	1 ml	Penicillin and Streptomycin
	1 ml	Glutamine
2). Hybridoma (HY) Medium	62 ml	Dulbecco's with $NaHCO_3$
	1 ml	HEPE's Buffer
	1 ml	Non-essential Amino Acids
	10 ml	NCTC 109
	20 ml	Fetal Calf Serum
	0.45 ml	Na Pyruvate
	1 ml	Bovine Insulin
	1 ml	Thymidine
	1 ml	Oxaloacetic Acid
	1 ml	Hypoxanthine
	1 ml	Penicillin and Streptomycin
	1 ml	Glutamine

3). Hypoxanthine/Aminopterin/Thymidine (HAT) Medium

A) 1XHAT	99 ml	HY Media

	1 ml	40 μM aminopterin
B) 2XHAT	98 ml	HY Media
	2 ml	40 μM aminopterin
4). CCL-130 Medium	75 ml	Leivowitz Medium
	1 ml	HEPE's Buffer
	1 ml	Non-essential Amino Acids
	20 ml	Fetal Calf Serum
	0.45 ml	Na Pyruvate
	1 ml	Glutamine
	1 ml	Penicillin and Streptomycin
5). Freezing Medium	80 ml	RPMI Medium with Antibiotics
	10 ml	Fetal Calf Serum
	10 ml	Dimethylsulfoxide (DMSO)

B. Fusion Reagents:

1) Dulbecco's with Fetal Calf	80 ml	Dulbecco's
	20 ml	Fetal Calf Serum
2) Dulbecco's serum free	99 ml	Dulbecco's
	1 ml	HEPE's Buffer
3) 30% Polyethylene Glycol	3 ml	Polyethylene glycol MW 1000
	7 ml	Dulbecco's serum free
4) NH_4Cl-EDTA	0.17 M	

C. Immunofluorescent screening:

1) Phosphate Buffered Saline (PBS) pH 7.4

2) Goat anti-Human IgG Fluorescein Conjugated Cappel Laboratory Centrifuged to remove unconjugated fluorescein and diluted 1/100 in PBS

3) Coverslipping Medium — 90% Glycerol

10% PBS

D. Avidin-Biotin-Peroxidase (Immunoperoxidase) Staining on Frozen Sections:

1) Phosphate Buffered Saline — pH 7.4

2) 0.03% H_2O_2 — 0.1 ml 30% H_2O_2

9.9 ml Methanol

3) 0.01% Avidin — 1 mg of Avidin dissolved in 10 ml PBS

4) 0.01% Biotin — 1 mg of Biotin dissolved in 10 ml PBS

5) Vectastain ABC Kit — Vector Laboratories

A) For Human IgG Detection — PK-4003

B) For Mouse IgG Detection — PK-4002

6) Chromogenic substrate — 5 mg diaminobenzidine tetrahydrochloride

5 ml TRIS/HCl buffer pH

10 µl 30% H_2O_2

5 ml deionized distilled H_2O

7) Counterstain: — Erhlich's Hematoxylin

E. Enzyme-Linked Immunosorbant Assay (ELISA) screen for human IgG production:

1) Phosphate Buffered Saline — pH 7.4

2) Alkaline Buffer — 50 mM Bicarbonate pH 9.6

3) 0.1% Crystalline Bovine Serum Albumin

4) Vectastain ABC Kit — PK-4003

5) Chromogenic Subtrate — 8 mg o-phenylenediamine dihydrochloride

10 ml 100 mM citrate/phosphate buffer

10 µl 30% H_2O_2

10 ml deionized distilled H_2O

F. ELISA Screen for murine anti-tumor antibody production:

1) Phosphate Buffered Saline	pH 7.4
2) Alkaline Buffer	50 mM Bicarbonate pH 9.6
3) 0.03% H_2O_2	0.1 ml 30% H_2O_2
	9.9 ml Methanol
4) 0.01% Avidin	1 mg Avidin dissolved in 10 ml PBS
5) 0.01% Biotin	1 mg Biotin dissolved in 10 ml PBS
6) Vectastain ABC Kit	PK-4002
7) Chromogenic Substrate	8 mg o-phenylenediamine dihydrochloride
	10 ml 100 mM citrate/phosphate buffer
	10 µl 30% H_2O_2
	10 ml deionized distilled H_2O

REFERENCES

REFERENCES

1. Simpson, E.: Antigens associated with H-2, embryos and tumors. Nature vol 306 22/29 December, 1983.

2. Hokama, Y. and Nakamura, R.: Immunology and Immunopathology, Little, Brown and Company, Boston, MA., 1982.

3. Bakerman, S.: Tumor Markers: Types and applications. Chem Cues, March 1984.

4. Wheelock, E. F. and Robinson, M.K.: Biology of disease, endogenous control of the neoplastic process. Lab Invest vol 48 no 2, 1983.

5. Eisen, H.: Immunology, Harper and Row Publishers, Philadelphia, PA, 1980.

6. Kohler, G. and Milstein, C.: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature vol 256 August, 1975.

7. Kennett, R. H., McKearn, T. J. and Bechtol, K. B.: Monoclonal Antibodies Hybridomas: A New Dimension in Biological Analyses, Plenum Press, New York and London, 1981.

8. Cute, R. J., Morrissey, D. M., Houghton, A.N., Beattie, Jr., E. J., Oettgen, H. F. and Old, L. J.: Generation of human monoclonal antibodies reactive with cellular antigens. Proc Natl Acad Sci USA vol 80 April, 1983.

9. Glassy, M. C., Handley, H. H., Lowe, H., Solol, R. E., Leonard, J. and Royston, I.: A human monoclonal antibody reactive against a human tumor-associated antigen. Fed Proc, 41:805, 1982.

10. Schlom, J., Wunderlich, D. and Teramoto, Y. A.: Generation of human monoclonal antibodies reactive with human mammary carcinoma cells. Proc Natl Acad Sci USA vol 77 no 11 November, 1980.

11. Collier, R. J. and Kaplan, D. A.: Immunotoxins. Sci Am vol 251 no 1 July, 1984.

12. Miller, R. A., Maloney, D. G., Warnke, R. and Levy, R.: Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. N Eng J Med vol 306 no 9 March, 1982.

13. Gordon, D. S.: Fast track for monoclonal antibodies. Med Lab Obs July, 1983.

14. Oldham, R. K.: Current status of monoclonal antibodies in cancer therapy. Clin Imm Newsletter vol 4 no 10 October, 1983.

15. Goldstein, G. and Sanders, M.: Monoclonal antibodies in clinical medicine. Clin Imm Newsletter vol 4 no 6 June, 1983.

16. Reizenstein, P.: Immunological and biological response modification in tumor treatment. Clin Imm Vnol Newsl vol 4 no 2 February, 1983.

17. Falini, B. and Taylor, C.: New developments in immunoperoxidase techniques and their application. Arch Pathol Lab Med vol 107 March, 1983.

18. Lusheng, S. and Whiteside, T. L.: Immuno phenotyping of lymphoid cells in tissues with monoclonal antibodies by avidin-biotin-peroxidase techniques. Clin Imm Newsletter vol 4 no 3 March, 1983.

19. Chien, L.: Fluorescent antibody techniques. Diagnostic Procedures For Viral And Ricketts Infection 4th ed.

20. Johnson, G. D. and Holbrow, E. J.: Immunology. Handbook of Diagnostic Clinical Immunology vol 1 Oxford, 1974.

21. DeLellis, R. A., Sternberger, L. A., Mann, R. B., Banks, P. M. and Nakane, P. K.: Immunoperoxidase technics in diagnostic pathology. A workshop by Am Soc Clin Path May, 1979.

22. Taylor, C. R.: Immunoperoxidase techniques. Arch Pathol Lab Med vol 102 March, 1978.

23. Giorno, R.: Characterization of mononuclear cells in cytocentrifuge and imprint preparations using monoclonal antibodies and an avidin-biotin immunoperoxidase staining system. J Histochem and Cytochem vol 31 no 11 1983.

24. Wood, G. S. and Warnke, R.: Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem and Cytochem vol 29 no 10 1981.

25. Teng, N. N. H., Lam, K. S., Riera, F. C. and Kaplan, H. S.: Construction and testing of mouse-human heteromyelomas for human monoclonal antibody production. Proc Natl Acad Sci USA vol 80 December, 1983.

26. Klausner, A.: Hopkins lab improves hybridoma production. Biotechnol September, 1984.

VITA

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