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There is evidence that the body's immune system continuously surveys for anomalous cells, such as malignant tumor cells, and destroys them before they become clinical cancers. The corollary to this principal is that clinical cancers are those cells that by some unknown mechanism escape immune surveillance. One similar benign condition already exists in humans: the tolerance of a fetus, whose genetic makeup is partially foreign, in a pregnant woman. My thesis was designed to test the hypothesis that this tolerance in pregnancy and in cancer, is the result of a shared set of cell surface antigens, which allow both types of cells to escape immune destruction.

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A SEARCH FOR ANTIGENS COMMON TO FETAL AND TUMOR CELLS

by
Joseph Bonn

A Thesis
Submitted in Partial Fulfillment
of the Requirements for the Degree with Honors
in Biology

WILLIAMS COLLEGE
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ABSTRACT

Contemporary research has focused on the intimate relationship between fetal and tumor cells, following the theory that the malignant transformation process is a reexpression of gene products of embryonic development. Through the use of *in vivo* and *in vitro* immunological methods, researchers have shown that malignant cells of a number of experimental types of cancer possess tumor-associated embryonic antigens. Investigators have been able to demonstrate immunologic cross-reactivity between embryonic-cell surface antigens and certain tumor cells.

Research conducted by this author attempted to demonstrate the presence of antigens common to fetal and tumor cells by one *in vivo* and three *in vitro* immunologic methods. Immunization with 13-day old fetal cells from a primiparous C3H mouse was found not to offer host protection from subsequent challenge by a transplanted fibrosarcoma originally induced *in vivo* by 3-methylcholanthrene. A complement-dependent microcytotoxicity assay performed in microtiter plates did not show cytotoxicity of fetal cells by anti-embryo-cell antiserum or by serum from pregnant multiparous C3H mice. A membrane immunofluorescence reaction showed qualitatively equivalent specific staining of fetal cells by anti-embryo-cell antiserum as by anti-adult-cell antiserum.

Cross-reactivity between fetal and tumor cells was shown by the demonstration of *in vitro* cytotoxicity of effector spleen

cells from a pregnant multiparous C3H mouse against cultured target tumor cells derived from a transplanted fibrosarcoma induced *in vivo* by 3-methylcholanthrene.

INTRODUCTION

Recent work in cancer research has focused on the multi-faceted relationship between the embryonic cell differentiation process and malignant cell growth, with the hope that common characteristics may reveal basic properties of tumor cells and provide a mechanism for specific killing of cancer cells. It has been well documented by both *in vitro* and *in vivo* experimentation that malignant cells of a large number of cancers of both immunogenic and non-immunogenic forms possess embryonic antigens (Baldwin and Vose, 1974; Artzt, 1973; Salinas and Hanna, 1974; Stonehill and Bendich, 1970). Alexander (1974) proposes three hypotheses to explain the presence of fetal macromolecules in tumor cells. The first theory involves the fortuitous synthesis of these macromolecules, a situation analogous to the synthesis of specific hormones by malignant tissue unrelated to the natural hormonal source tissue. The second theory interprets cancer as a disease of the differentiation mechanism, supporting the theory that tumors arise from embryonic cells which have been sequestered from the normal developmental patterns of their neighbor cells (Medawar, 1974). Knox (1974) postulates that tumors possibly arise from the dedifferentiation of adult cells. In either case, there would then exist an obligate association between the malignant transformation process and the presence on the tumor cell of fetal antigens. Alexander's third theory considers oncogenesis

as consisting of two major cellular changes: the first is the malignant transformation by which the cell becomes unresponsive to the normal controls on growth; this does not require the presence of fetal characteristics. The second cellular change according to Alexander is the proliferation of the transformed cells, which does necessitate fetal cell characteristics.

Anderson and Coggin (1974) examine theories on the genetics of cancer and cell differentiation and provide a possible interpretation for the embryonic antigens expressed on certain tumor cells. They explain that no singular genetic defect has yet to be found in a cancer cell; rather, there most likely exist multiple molecular changes as seen in many other diseases. The initiation of these changes may be postulated as a random clustering of accidents (by which mutant proteins would be synthesized), or as a change in the mechanisms which govern gene expression (whereby mutant proteins would not be synthesized). Since no abnormal cellular products have been detected in human cancer patients, the latter theory of alterations in the gene expression mechanisms as initiators of cancerous molecular changes would appear to be more likely. Concerning cell differentiation, Anderson and Coggin propose two theories of gene expression. By the additive theory, genes are activated in a linear sequence during differentiation, later cell stages expressing genes further along the sequence only. By the selective theory, genes are expressed in time-specific phases transiently during differentiation. If this latter theory is the more applicable explanation, then embryonic antigens

detected on cancer cells may be due to reactivation of normal genes that were time-specific for an embryonic developmental phase. It has also been postulated that fetal antigens may be present in a masked form on the normal adult cell, without eliciting immune recognition until they are uncovered in some manner by the malignant transformation (Ting, et al., 1972). Support for a theory by which characteristic cancerous molecular changes are shown to be the reexpression of the gene products of embryonic development is well documented (Baldwin, Glaves and Vose, 1972; Baldwin and Vose, 1974; Anderson, et al., 1974).

Detection of Tumor-Associated Embryonic Antigen

A number of studies have shown the presence of embryonic antigen on tumor cells for a wide variety of immunogenic and non-immunogenic tumors, and by a number of in vivo and in vitro methods. These share in common the demonstration of immunogenic cross-reactivity between this tumor-associated embryonic antigen (TAEA) and embryo cells, or embryonic antigen and certain tumor cells. Stonehill and Benditch (1970) have elaborated anti-embryonic-tissue antiserum which produced precipitin lines in a double diffusion assay to extracts of 72 different malignant tissues. Baldwin, Glaves and Vose (1972) have demonstrated surface embryonic antigens on carcinogen-induced rat hepatomas and sarcomas by a membrane immunofluorescence reaction between tumor cells and serum from multiparous females. Baldwin and Vose (1974) have also shown TAEA to be present on the non-immunogenic 2-acetylaminofluorene-induced mammary and ear duct

carcinomata by microcytoxicity and indirect membrane immunofluorescence assays in which multiparous rat serum reacts with tumor target cells *in vitro*. The non-immunogenic nature of this tumor relates to its inability to elicit a tumor rejection response in a syngeneic host (thus supporting the belief developed below that TAEA are distinct from tumor-specific transplantation antigens also present on tumor cell surfaces). Baldwin, Glaves and Vose (1974) have shown lymph node cells from multiparous females to be cytotoxic *in vitro* for plated target tumor cells. Baldwin and Vose (1974) have also shown the presence of TAEA in the converse manner by demonstrating lymph node cells and sera from tumor-immunized rats to be cytotoxic *in vitro* to cultured embryo cells.

Artzt, *et al.*(1973) have examined TAEA in a specific tumor, a teratocarcinoma which arose from neoplastic primordial germ cells in the male embryo. Antisera raised against primitive cells of this teratocarcinoma were shown to share surface antigens with cleavage stage embryos by specifically reacting only to the teratocarcinoma cells, male germ cells, and cleavage stage embryo cells. The authors suggest that there exists a progressive expression of surface antigens during embryonic development as demonstrated by the increased reactivity between the antisera and progressively older cleavage embryos from the one-cell stage through the eight-cell stage.

Salinas and Hanna (1974) have demonstrated *in vivo* cross-reactive host immunity by immunizing host animals with tumor cells to generate a cytostatic or cytotoxic host response to

transplanted fetal liver cells. By irradiating the hosts after tumor cell immunization, the authors attributed the cross-reactive host immunity to residual circulating antibody, since the irradiation destroyed other reactive immune system components.

The above demonstrations of tumor-associated embryonic antigens are supported by Knox (1974) whose bio-chemical studies show a prototypic tumor cell composition to be similar to many fetal tissues in quantitative patterns of enzymes and qualitative identification of certain isozymes.

Role of Tumor-Associated Embryonic Antigen in Tumor Immunogenicity

An examination of the role of TAEA in the immune properties of the tumor cell reveals widespread support of the hypothesis of Hanna, Tenant and Coggin (1971) that neo-plastic transformation of a cell causes derepression of fetal genes which then produce tumor-associated embryonic antigens, whereas the same neo-plastic transformation causes de novo synthesis of surface antigens specific to that tumor. This tumor-specific antigen (TSA) is shown below to be the antigen primarily responsible for tumor immunogenicity in the host rejection response. In virally-induced tumors the TSA is common to all tumors induced by one particular virus type. However, in carcinogen-induced tumors, each induction with the same carcinogen is thought to produce a unique and different TSA.

Absorption tests by Baldwin, Glaves and Vose (1972) provide evidence for the distinction between TAEA and TSA. The authors have demonstrated that although anti-embryonic-cell antiserum

could be neutralized by absorption with a variety of tumors and fetal tissues, sera from tumor-immunized animals could not be neutralized by absorption with fetal tissue. These results suggest that there exists a common embryonic antigen (TAEA) in the many types of tumors used, but separate, distinct tumor-specific antigens. Further work by these authors (Baldwin, Glaves and Vose, 1974a) supports these results by showing TAEA and TSA are different on chemically-induced rat hepatoma and sarcoma in blocking-antibody studies. They demonstrated that serum from multiparous females protected in vitro against the cytotoxic effects of multiparous female lymph node cells for tumor and fetal cells. However, serum from multiparous females did not protect against the cytotoxicity of lymph node cells from tumor-immune rats for the immunizing tumor. In addition, the authors performed cell fractionation techniques, and by absorption tests on these subcellular fractions, they found TAEA to be located in the soluble cytoplasmic protein ("cell sap") as well as in the plasma membrane, whereas TSA were found associated only with the plasma membrane.

Ting, et al. (1972) have performed similar absorption studies, protecting against anti-embryo antiserum complement-dependent cytotoxicity for virally-induced tumor cells by absorption of the antiserum with tumor or embryo cells. Virally-induced-tumor-bearer serum lost its cytotoxicity only when absorbed with tumor cells transformed by that specific virus.

Brawn (1970), and Hellstrom and Hellstrom (1975a) have demonstrated lymph-node and spleen cells from pregnant, multiparous female mice to be cytotoxic in vitro to cultured syngeneic tumor cells, as compared to control fibroblast or non-

transformed cells. These cultured turmors include neoplasms induced in vivo by chemical carcinogens. The lymphocytotoxicity thus shown has been blocked by incubation with serum from either pregnant mice or mice carrying 3-methylcholanthrene-induced tumors (Hellstrom and Hellstrom 1975a). The authors have also removed the blocking ability of pregnant mouse serum by absorption of the serum with either neoplastic or fresh embryonic cells. These results demonstrate again the presence of tumor-associated embryonic antigens on chemically-induced, transplanted tumors. It has been demonstrated that tumor transplantation resistance could be obtained by immunization with that same tumor, but could not be elicited by immunization with embryonic tissue (Hellstrom and Hellstrom 1975b) or by in vivo adoptive transfer of multiparous female lymphocytes (Baldwin and Embleton 1974). This inconsistency of results, in which in vitro lymphocytotoxicity of tumor cells has not been shown in vivo, has provoked continuing research to distinguish the properties of TAEA and TSA.

Baldwin, Glaves, and Vose (1974a) have put forth a number of hypotheses to explain the lack of a role for tumor-associated embryonic antigens (TAEA) in eliciting tumor rejection. Their first postulation is of a humoral blocking-antibody response to TAEA preventing cellular immunity from being effective against the tumor-specific antigens. Secondly, TAEA may be unstable at the plasma membrane, become shed from the cell as non-immunogenic soluble antigen or immune complexes which could then block cellular immunity to TSA. Thirdly, as demonstrated, TAEA differ from TSA in cell location; perhaps this difference prevents

TAEA from assuming a role in effecting a tumor response.

An important consequence of the above results distinguishing TAEA from TSA is that *in vitro* lymphocytotoxicity assays for humans may be demonstrating the presence of TAEA rather than truly reflecting the *in vivo* tumor rejection capabilities of the patient.

Fetal-Cell Immunization and Resistance to Oncogenesis

Fetal antigen research has also been performed to evaluate the capability of fetal cells to elicit a cross-reactive immune response specifically inhibitory to cancer development or cytotoxic to existing tumor cells. A number of researchers have attained successful protection against a variety of tumor challenges by prior fetal cell immunization: Coggin, *et al.* (1971) with SV40-induced tumors, Girardi, *et al.* (1973) with adenovirus-31-induced tumors, Hanna, Tenant and Coggin (1971) with Rauscher virus-induced leukemia and a plasma cell ascites, and Castro, *et al.* (1974) with a transplanted Meth A tumor. Castro, *et al.* (1974) also prolonged the induction time of a 20-methylcholanthrene-induced tumor by prior inoculations with fetal tissue. However, inoculations with fetal tissue during the induction period of a tumor enhanced tumor growth. Failure has been almost as prevalent: Ting (1968) could not elicit protection against polyoma virus-induced tumors by prior fetal-cell immunization; Baldwin, Glaves and Vose (1974b) could not protect against 3-methylcholanthrene-induced tumor challenge. Parmiani and Lembo (1974) repeated this failure to protect against 3-methylcholanthrene-induced fibrosarcoma by prior

inoculation with x-irradiated or mitomycin-C blocked syngeneic or allogeneic primiparous embryo cells. In fact, the researchers produced enhancement of fibrosarcoma growth by inoculating males with embryonic tissue, by challenging multiparous females with tumor, by prior inoculation with syngeneic cells in Freund's adjuvant, or by inoculating with embryomas or syngeneic embryo cells in cell-permeable diffusion chambers. This enhancement was demonstrated to be immunologic in nature upon reduction by immunosuppression. Basombrio and Prehn (1972) support this data by demonstrating a lack of protection against methylcholanthrene-induced tumor after pregnancy or pre-immunization with embryonic tissue. The authors then performed splenectomies on multiparous female mice challenged with tumor in order to remove blocking antibody as a factor, and still demonstrated a lack of protection from the tumor.

Coggin, Ambrose and Anderson (1970) have shown the importance of using irradiated or mitromycin-C blocked embryo cells in the inoculum. Non-irradiated or unblocked embryo cells do not offer the protection from SV40 tumor cells that irradiated or blocked embryo cells were shown to have. The authors postulate that rapid maturation of the non-irradiated or unblocked embryo cells in the mature environment of the adult recipient causes these cells to lose that antigenicity which is effective in inducing protection from the tumor. This antigenicity is short-lived in the embryo cell itself; the authors demonstrated protection from SV40 tumor cells with inoculations of embryo cells only between the embryo age of 9 and 14 days.

Girardi, et al. (1973) have investigated the effect of parity status of females donating embryonic tissue for immunization and found that embryonic tissue from primiparous females protected against SV40-induced tumor challenge, whereas multiparous embryonic tissue did not offer protection. The researchers found a non-cytotoxic humor antibody, present only in pregnant multiparous females, which was reactive to SV40-induced tumor cells. This may be a blocking-antibody effect incapacitating the natural cellular immune response against the tumor cells (and may also be a method by which the embryo escapes the immune response of its mother). The researchers postulate that in primiparous embryonic tissue, the blocking-antibody titer would be too low, and therefore protection against the tumor is offered. In the multiparous embryonic tissue, sufficient blocking-antibody from the mother prevents protection against tumor challenge.

This phenomenon may be important in relation to the mode by which embryonic tissue escapes the maternal immune system, and the possible sharing of this escape mode by tumor cells possessing tumor-associated embryonic antigens. Alexander (1974) notes that anatomical barriers are insufficient means of escape from host immunity for both tumor and fetal cells, especially the latter. The placenta allows some maternal antibody to cross into the fetus (and vice-versa), and with embryo age the placenta becomes more "leaky" to cellular crossing. Since the fetus must therefore be exposed to both the maternal humoral and cellular responses, it must develop a method of escaping both. Alexander

disputes the postulated role of antibodies or antigen-antibody complexes as blocking agents allowing fetal or tumor cell escape, and instead proposes that the tumor-specific antigen sheds in a soluble form and then binds with cytotoxic antibodies and cells to render them ineffective. Soluble antigens are poor immunogens, as well, and would not elicit tumor rejection responses themselves. Alexander notes that antigen shedding may possibly be the result of immune system killing of tumor cells.

Having noted that to this date immunization of a host animal with embryonic cells prior to challenge with most chemically induced transplanted tumors has not offered the host protection from the tumor, and noting that each chemically induced tumor possesses individually distinct transplantation antigens, we first investigated the ability of an embryonic-cell immunization to protect against a 3-methylcholanthrene-induced fibrosarcoma originating in this laboratory.

Further work centered upon raising anti-embryonic-cell antiserum and testing *in vitro* this antiserum, serum from multiparous, pregnant females, and serum from mice bearing a methylcholanthrene-induced fibrosarcoma. We used two microcytotoxicity assays, hoping to detect specific complement-dependent cytotoxicity of embryo cells by these three sera. This anti-embryonic-cell antiserum was then tested *in vitro* against embryo cells in a membrane immunofluorescence assay.

The final work of this project was an *in vitro* testing of the lymphocytotoxic properties of spleen cells from multiparous, pregnant mice against cultured tumor cells derived from

a methylcholanthrene-induced fibrosarcoma. We used a cell-mediated microcytotoxicity assay to investigate this aspect of fetal-antigenic cross-reactivity with the tumor cells.

MATERIALS AND METHODS

Fetal-Cell Immunization

Protection from a 3-methylcholanthrene-induced transplanted tumor was attempted by a single inoculation of fetal cells from a primiparous female prior to challenge with the tumor.

Embryos were raised by timed matings between C3H male mice and virgin C3H female mice (Jackson Laboratories Bar Harbor, Maine, then bred in our laboratory). At the embryo age of 13 days (post detection of a copulation plug in the mother) the fetal cell inoculum was prepared. The mother was sacrificed, her embryos dissected out, placed in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) in a Petri plate, decapitated and minced with scissors to form a tissue suspension in the RPMI-1640. This suspension was then forced through a fine-mesh screen to further disperse cells. After a two-minute settling of larger tissue particles in a test tube, the supernatant was poured into a centrifuge tube, aspirated with a 3 cc. syringe and 20-gauge needle (Becton-Dickinson, Rutherford, N.J.), and then aspirated with a 23-gauge needle. The cell suspension was then washed twice with RPMI-1640 and counted on a hemacytometer, using exclusion of 0.2% trypan blue (National Aniline and Chemical Co., Inc., New York, N.Y.) to determine cell viability.

A suspension of fetal cells in RPMI-1640, at a concentration of 4.5×10^6 live cells/ml was prepared and given by suprascapu-

lar subcutaneous injection to ten C3H male mice. A suspension of adult male C3H mouse liver, lung, kidney and spleen was prepared in a manner similar to the fetal cell inoculum, and given at a concentration of 4.5×10^6 live cells/ml by supra-scapular subcutaneous injection to ten C3H male mice to serve as controls.

Three weeks after inoculations, these ten experimental and ten control mice were given a second generation transplanted tumor, originally induced by 3-methylcholanthrene. The challenge tumor cell suspension was prepared by removing second generation fibrosarcomas from two female C3H mice, mincing the tumors in RPMI-1640, processing the minced tissue with 0.25% trypsan (GIBCO, Grand Island, N.Y.) and crystalline deoxyribonuclease (National Biochemicals Corp., Cleveland, Ohio). The resultant cell suspension was filtered through a cotton gauze, the filtrate washed thrice with RPMI-1640, filtered once more through gauze and counted on a hemacytometer using exclusion of 0.2% tryspan blue to determine cell viability. The ten experimental and ten control mice were then each given 5.0×10^5 live tumor cells by subcutaneous injection. These animals were monitored for life span after tumor challenge.

Complement-Dependent Microcytotoxicity Assays

Four types of mouse serum were tested for presence of anti-embryo-cell antibody, by performing complement-dependent microcytotoxcity assays against target fetal cells from pregnant multiparous female mice. The sera were: anti-embryonic-cell antiserum, serum from pregnant multiparous mice, serum from fibrosarcoma-

bearing mice, and normal serum.

The first serum to be tested was prepared in C3H male mice by two separate inoculations of 13-day old fetal cells from primiparous C3H female mice. The first subcutaneous inoculation of 2.5×10^6 fetal cells was given to 10 C3H male mice; these mice received a second subcutaneous inoculation of 2.5×10^6 fetal cells 23 days later. Nine days later five experimental animals were sacrificed and their serum harvested. This was accomplished by deep etherization of the mouse, throat vessel sectioning, collection of blood in a test tube, removal of the clotting proteins by allowing a clot to form around an inserted wooden stick and freezing of the serum supernatant after high-speed centrifugation to remove cellular constituents (Coleman centrifuge, Coleman Instruments, Inc., Maywood, Illinois).

The second serum to be tested was harvested in the same manner from multiparous C3H female mice that were pregnant at the time of harvesting.

The complement-dependent microcytotoxicity assay was performed in microtiter plates using fetal cells from pregnant multiparous C3H female mice (which also donated the above serum) as target cells, and the above prepared anti-embryo cell anti-serum and multiparous female serum as separate effector sera. Each row of the microtiter plate contains ten wells, 25 µl of RPMI-1640 buffer was placed in all but the first well with an automatic pipette (Biopette, Schwarz/Mann, Orangeburg, N.Y.). The effector serum was then placed in the first and second wells, 25 µl in each with the automatic pipette. The serum-buffer

mixture was titrated with a microtiter rod from wells two through ten. At this point 25 µl of a 5.0 x 10^6 cells/ml concentration of fetal cells was added to all wells with the automatic pipette. This delivered, therefore, 1.25 x 10^5 fetal cells per test well. The automatic pipette was then used to add 25 µl of Guinea Pig Complement (Gibco Diagnostics, Madison, Wisconsin) to all wells. The microtiter plate was incubated at 37° for 45 minutes, covered with plastic sealing tape. After incubation, 50µl of 0.2% trypan blue as added to all wells; the plate was kept at room temperature for five minutes and then placed on iee. Each well was then individually aspirated with a Pasteur pipette and the cell suspension counted on a hemacytometer. Viable fetal cells exclude trypan blue, whereas dead cells acquire a light blue color and lack the dark circular membrane boundary which distinguishes the living cell.

In order to establish the validity of the complement-dependent microcytotoxicity procedure in our laboratory, the assay was first attempted using target thymus cells from a 4-week old C57 male mouse (at a concentration of 1.25 x 10^5 cells per well), and anti-theta antiserum as the effector serum. The assay was then repeated using target fetal cells; with anti-embryo-cell antiserum and pregnant multiparious female mouse serum as the separate effector serums. In this assay a control row of wells was prepared containing only buffer, complement and target fetal cells, thereby lacking an effector serum.

The complement-dependent microcytotoxicity assay was modified so that a Falcon 3034 Microtest plate (Falcon Plastics,

Oxnard, California) would take the place of the larger microtiter plate, titration would not be necessary, and the third and fourth sera would be used as well. In addition, the problem of evaporation of the microtiter well contents would be prevented by covering the contents with peanut oil.

The procedure for the Falcon microtest assay was fairly similar to that used with the microtiter plate. In contrast, however, buffer was not used in the Falcon microtest assay, and titration of the serum was not performed. In the microtest assay, peanut oil (I. Rokeach and Sons, Inc., Farmingdale, N.J.) was first added to all wells, a small droplet into each by Pasteur pipette. Water soluble components of the assay, such as complement or serum, added after this sank to the bottom of the well making the peanut oil a protective cover from evaporation. Effector serum was added to all wells in the row, 20 µl per well, with a Hamilton Microliter Syringe (Hamilton Co., Reno, Nevada). Guinea Pig Complement (20 µl) was added by the syringe, then 20 µl of target fetal cells from a pregnant multiparous C3H female. The fetal cell suspension was 5.0×10^6 cells/ml, therefore the syringe delivered 1.0×10^5 cells per well. The plate was incubated at 37 degrees Centigrade for 30 minutes, and then 40 µl of 0.2% trypan blue was added to all wells by microliter syringe. An inverted Unitron microscope was used to determine cell viability (live cells excluding trypan blue). The entire well could be viewed at once at 10x power; the design of the microtest plate, with all cells resting on the well bottom, necessitated the inverted microscope in order to focus close

enough without disturbing the well contents.

The microtest assay was performed using pregnant multiparous C3H mouse fetal cells as target cells, with four different effector sera. The first and second sera as described above were the anti-embryo-cell antiserum and serum from the pregnant multiparous C3H female, respectively. The third was serum taken from a male C3H mouse bearing a transplanted fibrosarcoma originally induced by 3-methylcholanthrene. The fourth effector serum was a control serum taken from a normal C57/bl mouse.

Immunofluorescence

Anti-embryo-cell antiserum, prepared by double inoculations of a C3H mouse with a primiparous female's fetal cells, was tested for presence of anti-embryo-cell antibody by a membrane immunofluorescence reaction. The presence of this antibody, bound to fetal target cells after the two had incubated together, would be detected by the binding of fluorescein-labelled anti-IgM (anti-mouse-immunoglobulin) (Cappel Laboratories) to the tested antibody bound to the target fetal cell. Upon examination under a fluorescent microscope, each cell with bound anti-embryo-cell antibody and anti-IgM would fluoresce qualitatively brighter than those cells lacking bound antibody and fluorescent anti-IgM.

A suspension of 13-day old primiparous female's fetal cells in RPMI-1640 was prepared as described above. A 0.1 ml aliquot of this suspension (or, approximately 1.5×10^6 cells) was added to 0.1 ml of anti-embryo-cell antiserum, prepared as

above by double inoculation of a C3H mouse with primiparous female's fetal cells. For a control test, the anti-embryo-cell antiserum was replaced by anti-adult-cell antiserum, prepared by double inoculation of a C3H mouse with adult cells (including liver, spleen, kidney and lung). The suspension of target fetal cells and antiserum was incubated for 25 minutes at room temperature, washed twice with Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO, Grand Island, N.Y.) and then resuspended in PBS. This period allowed any specific antibody to bind with the target fetal cells, while washing away any unbound antibody. Fluorescent anti-mouse immunoglobulin (anti-IgM) was then added, 0.1 ml. The mixture of fetal cells, any bound antibody, and fluorescent anti-IgM was then incubated at room temperature for 25 minutes to allow any possible binding of anti-IgM to the fetal-cell-bound antibody, and then washed twice with PBS to remove any unbound fluorescent anti-IgM. A Pasteur pipette was then used to place one drop of this mixture on a glass slide, to which one drop of warmed (37 degrees Centigrade) Elvanol was added and stirred in to fix the cells and the glass cover slip. The prepared slide was then dried in darkness overnight. A qualitative comparison under the fluoresecent microscope was made between the slide with anti-embryo-cell antiserum and the slide with anti-adult-cell antiserum, as to the extent and brightness of fluorescence.

Cell-Mediated Microcytotoxicity

Spleen cells from a pregnant multiparous C3H mouse were tested for their cross-reactivity with tumor cells by a microcytotoxicity assay involving effector spleen cells and target

cultured tumor cells derived from a 3-methylcholanthrene-induced fibrosarcoma. This procedure necessitated culturing the fibrosarcoma target cells from a transplanted tumor, adding the effector spleen cells with a culture media solution, incubating this mixture two days and determining the number of tumor cells adhering to the well bottoms of the culture plate. All procedures, therefore, were performed aseptically in order to prevent contamination of the culture media during the tumor culturing and incubation with the effector cells.

In the first trial of this assay, a twelfth generation transplanted tumor was removed with sterile instruments from a male C3H host. The tumor was placed in RPMI-1640 in a sterile Petri plate, minced with sterile scissors; the mixture was then placed in a bottle with an equal amount of trypsin and deoxyribonuclease and stirred for 45 minutes. At all steps in the procedure sterile technique was maintained with sterile solutions and instruments. The mixture was filtered through gauze to remove large tissue pieces, and the filtrate was washed four times with RPMI-1640. Viable cells were counted in a hemacytometer using exclusion of 0.1% trypan blue as an indicator of viability. The tumor cell suspension was then diluted to a concentration of 1.5×10^5 cells/ml in a culture media prepared as follows. Ten ml of media is composed of 6.6 ml RPMI-1640, 3.0 ml Fetal Calf Serum (GIBCO, Grand Island, N.Y.), 0.15 ml Antibiotic-Antimycotic (GIBCO), and 0.15 ml Anti-PPLO (Tylocine, 6,000 mg/ml) (GIBCO). This tumor cell suspension was then delivered by an Eppendorf Micro Pipet (Brinkmann Instruments, Inc.,

Westbury, N.Y.), ten µl per well (1,500 cells per well) into all wells of a Falcon 3034 microtest tissue culture plate. The plate was incubated overnight to allow *in vitro* tumor cell growth.

The effector spleen cells were prepared the next day, just prior to removal of the microtest plate from the incubator. The experimental spleen cells were taken from pregnant multiparous C3H mice, whereas the control spleen cells were harvested from normal 8-week old C3H female mice. At all times during the processing of spleen cells, sterile technique was maintained as closely as possible.

After removal of the spleen into RPMI-1640 in a Petri plate, the spleen was dissected apart using an 18-gauge hypodermic needle. The mixture was placed in a test tube to allow large tissue particles to settle out, and the supernatant was poured off to be aspirated with a 3 cc syringe and 20-gauge needle, followed by the same syringe and a 23-gauge needle. The cell suspension was washed twice with RPMI-1640, counted for viable cells by 0.1% trypan blue exclusion on a hemacytometer, and diluted in the culture media prepared above to concentrations of 5.0×10^6 cells/ml, 2.5×10^6 cells/ml and 1.0×10^6 cells/ml, in capped plastic test tubes (Falcon 2003 tube, Falcon Plastics, Oxnard, California).

The cultured tumor cell plate was then removed from the incubator and each well washed with culture media by dumping out the well contents and dropping in new media with a Pasteur pipette, thus effectively removing dead tumor cells which do not

adhere to the well bottom. The plate was briefly examined under the inverted microscope to determine the extent of tumor cell viability in each well before proceding with addition of the effector spleen cells. Viable cultured tumor cells will adhere to the plastic bottom of each test well and form long dendritic extensions with which to secure themselves, and thus are easily detected.

The effector spleen cells were then added by Micro pipet using sterile pipet tips, 10 µl of cell suspension in each well. Therefore, the test concentrations were 5.0×10^4 effector spleen cells against 150 target tumor cells (the approximate survival ratio for the tumor culturing was 10% of 150,000 cells delivered), 2.5×10^4 effector spleen cells against 150 target tumor cells, and 1.0×10^4 effector spleen cells against 150 target tumor cells. The effector cell, target cell mixture was incubated for two days. Upon removal from the incubator, all wells were washed twice with PBS, stained with Giemsa stain (E. F. Mahady Co., Boston, Mass.) for 40 minutes, rinsed with distilled water and air dried. Tumor cells were counted in each well with the inverted microscope, and an average value for the six wells of each row was calculated.

The second trial of the cell-mediated microcytotoxicity assay utilized the same concentrations of effector and target cells, however, the thirteenth generation of the same transplanted tumor line was used for target cells. In the third trial, the thirteenth generation tumor was used for target cells, however, the target cell concentration was doubled prior to addition of

the effector spleen cells, thereby providing approximately 300 target tumor cells per well.

RESULTS

Fetal-Cell Immunization

A single inoculation of 13-day old fetal cells from a primiparous C3H female mouse failed to elicit protection from challenge by a transplanted fibrosarcoma, originally induced *in vivo* by 3-methylcholanthrene, as compared to control mice inoculated with adult cells prior to challenge with the same tumor.

Complement-Dependent Microcytotoxicity Assays

The complement-dependent microcytotoxicity assay performed in microtiter plates was shown to be valid by demonstrating significant cytotoxicity of thymus cells by anti-theta antiserum and Guinea Pig Complement, as compared to control tests involving only thymus cells and complement.

The first serum tested in this assay was anti-embryo-cell antiserum prepared by double inoculation of C3H male mice with 13-day old fetal cells from primiparous female mice. With this antiserum we were unable to demonstrate cytotoxicity of a multiparous C3H mouse's fetal cells significantly greater than that shown in control tests involving only the fetal cells and complement.

The second serum tested in the microtiter plate assay was serum from pregnant multiparous C3H mice, harvested for each test from the same mouse which supplied the target fetal cells. With this antiserum we were unable to demonstrate cytotoxicity

of fetal cells significantly greater than that shown in control tests involving only the fetal cells and complement.

The complement-dependent microcytotoxicity assay performed in the Falcon Microtest plates could not be perfected to produce visible results when testing cytotoxicity of fetal cells by anti-embryo-cell antiserum, serum from a pregnant multiparous C3H mouse, serum from a male C3H mouse bearing a transplanted fibrosarcoma originally induced in vivo by 3-methylcholanthrene, and serum from a normal C57/bl mouse. The assay was repeated using target thymus cells, effector anti-theta antiserum, and Guinea Pig Complement, and still could not be perfected to produce visible, conclusive results.

Immunofluorescence

Anti-embryo-cell antiserum, tested for presence of anti-embryo-cell antibody by a membrane immunofluorescence reaction, produced specific fluorescent staining (of fetal cells from a primiparous C3H mouse) qualitatively equivalent to that produced by anti-adult-cell antiserum.

Cell-Mediated Mycrocytotoxicity

The first trial of the cell-mediated microcytotoxicity assay produced a low plating efficiency of tumor cells, as shown by a low viable tumor cell count in the control wells containing culture media and no effector spleen cells, and as repeated in the erratic plating in the experimental wells. The results of this trial were therefore determined to be inconclusive.

The second trial of this assay also showed a low tumor-

DISCUSSION

The failure to elicit transplantation resistance to a transplanted fibrosarcoma (which was originally induced *in vivo* by 3-methylcholanthrene) in a host previously inoculated with 13-day old fetal cells from a primiparous C3H mouse is consistent with the majority of results achieved by other researchers working with chemically induced tumors (Baldwin, Glaves and Vose 1974b; Parmiani and Lembo 1974; Basombrio and Prehn 1972; Hellstrom and Hellstrom 1975b). This is in contrast to the primarily successful protection elicited against virally-induced tumors as mentioned in the Introduction. However, there are exceptions with both kinds of tumors; Castro, *et al*. (1974) has shown protection against primary tumors induced by 20-methylcholanthrene, whereas Ting (1968) failed to protect against tumors induced by polyoma virus. In the majority of cases, it may be that chemically induced tumors more rapidly lose their tumor-associated embryonic antigens in the process of transplantation after original induction. Another possible explanation for our inability to elicit protection may be the timing of the fetal cell inoculation and the tumor cell challenge; the time of tumor challenge may not have corresponded to the maximum immune reactivity produced by the fetal cell inoculation. These questions remain unresolved in this area of research.

Complement-dependent microcytotoxicity assays performed in the microtiter plates were designed to demonstrate the presence

cell plating efficiency in control wells containing culture media and no effector spleen cells. However, in those experimental wells treated with effector cells from a pregnant multiparous C3H mouse, possible enhancement of cultured target tumor cell growth was demonstrated when compared to the viable tumor cell count in those wells treated with effector spleen cells from a normal C3H mouse. This raises the possibility of immunostimulation, which will be described in the Discussion section below.

The third trial of the assay showed a high tumor-cell plating efficiency in the control wells treated with culture media and no effector spleen cells. Analysis by a parametric, unpaired t-test (Steel and Torrie 1960) of data from wells receiving 5.0×10^4 effector spleen cells per 150 target tumor cells showed statistically significant cytotoxicity of target tumor cells by effector spleen cells from pregnant multiparous C3H mice as compared to effector spleen cells from normal C3H mice (Table 1). Analysis of the data from wells receiving 2.5×10^4 effector spleen cells per 150 target tumor cells did not show statistically significant cytotoxicity (Table 2). The low tumor plating efficiency in those wells treated with 1.0×10^4 effector spleen cells may have contributed to these results being inconclusive.

of anti-embryo-cell antibody in serum from C3H mice inoculated with primiparous fetal cells and serum from multiparous C3H mice. After positive display of anti-embryo-cell antibody, the test would have been extended to demonstrate possible cytotoxicity of this antibody against target tumor cells, thereby demonstrating cross-reactivity between fetal and tumor antigens. The failure of the microcytotoxicity assay for these two sera and the failure of the membrane immunofluorescence reaction in detecting anti-embryo-cell antibody precluded testing against target tumor cells.

Results of the second trial of the cell-mediated microcytotoxicity assays, in which effector spleen cells from pregnant multiparous C3H mice appeared to enhance cultured target tumor cell growth as compared to those tumor cells treated with effector spleen cells from normal C3H mice, suggests that immunostimulation may have been a factor in the assay. Research by Prehn (1972) on immunostimulation suggests that there is a dual *in vivo* immune response to a tumor challenge; early in the malignancy stimulation of tumor growth may occur due to a low-level immune reaction. Later in the malignancy, the increased immune response may cause inhibition of tumor growth. Prehn has shown that small numbers of lymphocytes from tumor-bearing animals, injected simultaneously with tumor cells (to which these lymphocytes were cytotoxic *in vitro*) into normal or immunodeficient mice produced enhancement of tumor growth. On the other hand, large numbers of immune spleen cells injected with tumor cells produced inhibition of tumor growth. The results of Fidler (1973) and Carnaud, *et al.* (1972) support Prehn's work: a cell-mediated response cytotoxic

to tumor cells *in vitro* proves to be stimulatory at a lower level *in vivo*.

Other researchers have shown that tumor growth may be stimulated by a low-level immune response to an unrelated antigen as well. Ilfeld, *et al.* (1973) showed that lymphocytes sensitized against fibroblast (muscle) cells stimulate a low-level immune response which then seems to be responsible for enhancement of tumor growth. Lymphocytes sensitized against either the fibroblast cells or 3LL tumor cells were equally cytotoxic *in vitro* against the 3LL tumor, but *in vivo* both types of sensitized lymphocytes promoted 3LL tumor growth. Paranjpe and Boone (1974) showed that lymphocytes sensitized to tuberculin cells were the source of a low-level immune response which in turn stimulated growth of SV-40-transformed and methylcholanthrene-induced fibrosarcomas when a mixture of tuberculin and tumor cells was given to a tuberculin-immune host.

The quantification of the low-level immune response thought responsible for enhancement of tumor growth remains difficult to define. Whereas Prehn found a 100:1 ratio of immune lymphocytes to tumor cells protects the host against a tumor challenge, and a 1:1 ratio enhances tumor growth, Ilfeld found that the 100:1 ratio of immune lymphocytes to tumor cells enhanced tumor growth.

Spleen cells from pregnant multiparous C3H mice may be providing the low-level cell-mediated immune response *in vitro* that may contribute to enhancement of the target tumor cell growth in the second trial of the microcytotoxicity assay. The low plating efficiency seen in control wells receiving culture media and no effector spleen cells, and the subsequent cytotoxi-

city demonstrated in the third assay identical to this favor interpreting these apparent tumor growth enhancement results as laboratory artifacts.

The statistically significant cytotoxicity of effector spleen cells from pregnant multiparous C3H mice against target tumor cells correlates with similar results obtained by Brawn (1970) and Hellstrom and Hellstrom (1975a). We have thereby been able to statistically demonstrate *in vitro* the presence of tumor-associated embryonic antigens on a cultured transplanted tumor originally induced *in vivo* by 3-methylcholanthrene. However, like most investigators, we have not been able to utilize fetal cell cross-reactivity with tumor-associated embryonic antigen to protect *in vivo* the fetal-cell immunized host from a subsequent chemically induced transplanted tumor challenge. The frequent inability to demonstrate protection from tumor challenge after immunization with fetal cells (Hellstrom and Hellstrom, 1975b) and the failure to establish tumor transplantation resistance by *in vivo* adoptive transfer of lymphocytes from multiparous female mice (Baldwin and Embleton 1974) are paradoxes yet unresolved and most likely the focus of continuing research into understanding the nature of fetal and tumor cell antigens.

Table 1

Cell-Mediated Microcytotoxicity: Trial 3
Cytotoxicity of cultured tumor cells by 5 x 10^4 pregnant multiparous spleen cells

Percentage of tumor cells in spleen-cell-treated wells per average tumor cells in untreated wells

(Treated with 5 x 10^4 effector spleen cells from a pregnant multiparous C3H mouse)		(Treated with 5 x 10^4 effector spleen cells from a normal C3H mouse)	
	45.1		71.8
	40.1		81.0
	39.4		97.2
	43.0		86.6
	78.9		135.2
	49.3		73.9
Mean	49.3*	Mean	91.0*
S.E.	±6.1	S.E.	±9.6

*there is a significant difference between these columns of figures ($p<.05$)

Table 2

Cell-Mediated Microcytotoxicity: Trial 3
Absence of tumor cell cytotoxicity by 2.5×10^4 pregnant multiparous spleen cells

Percentage of tumor cells in spleen-cell treated wells per average tumor cells in untreated wells

(Treated with 2.5×10^4 effector spleen cells from a pregnant multiparous C3H mouse)	(Treated with 2.5×10^4 effector spleen cells from a normal C3H mouse)
63.4	58.5
51.4	90.1
73.9	95.8
99.3	123.2
73.9	94.4
72.5	62.7
81.7	41.5
62.0	96.5
54.2	57.0
57.7	145.1
62.0	98.6
52.8	48.6
Mean 67.1*	Mean 79.6*
S.E. ±4.0	S.E. ±8.1

*there is not a significant difference between these columns of figures ($.05<p<.10$)

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