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13. ABSTRACT *(Maximum 200 words)*

The purpose of this research is to develop an autologous breast cancer vaccine whose immunogenicity we hypothesize will be significantly enhanced by the ectopic expression of the human T cell co-stimulatory molecule, B7-1. In the past year, the feasibility of short-term culture of human breast cancer cells harvested from metastatic sites has been clearly demonstrated. Incubation of these human breast cancer cells with the adenoviral expression vector, Ad.hB7, resulted in greater than 90% of the cells expressing B7-1 protein on their surface. This high level of expression persisted for at least one month. These results demonstrate that the experimental approach that will be utilized in the clinical trial is technically feasible. Experiments conducted in two different mouse mammary carcinoma models support the hypothesis that B7-1 expression enhances the immune recognition of tumor cells; however, both models also indicate additional cytokines (i.e. IL-12 or GM-CSF) that might improve the immune mediated regression of tumors and will be studied further in the next year with the anticipation that they may be added to the upcoming clinical trial.

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John W. Smith Sr. 10/28/96

PI - Signature Date

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Annual Report

DAMD17-94-J-4385

"Gene Therapy of Human Breast Cancer"

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Attached manuscript (in press, *Cancer Gene Therapy*): Immune Responsiveness to a Murine Mammary Carcinoma Modified to Express B7-1, IL-12 or GM-CSF. E. Aruga, A. Aruga, M. Arca, W. Lee, N. Yang, J.W. Smith, A. Chang. (1996) Appendix 4

I. Introduction

The purpose of this research is to develop an autologous breast cancer vaccine whose immunogenicity we hypothesize will be significantly enhanced by genetic modification with an adenoviral expression vector for the T cell co-stimulatory molecule, B7-1.

The technical objectives of this research as outlined in the original proposal are to:

1. Develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer.

2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells used as a vaccine to enhance the immune response to the tumor.

4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

II. Body

Brief history of the grant

This research project proposal was submitted by the University of Michigan to the US Army Medical Research and Materiel Command in November 1993. It was approved in May 1994 and funding started in October 1994. The principal investigator was John W. Smith II, M.D., then an Associate Professor in the Department of Internal Medicine at the University of Michigan involved in clinical research in breast cancer and immunotherapy. Associate investigators included Laurence A. Turka, M.D., then an Associate Professor in the Department of Internal Medicine, Director of the University of Michigan Cancer Center Tumor Immunology Program and recognized expert in T cell costimulation pathways, Stephen P. Ethier, Ph.D., Assistant Professor in the Department of Radiation Oncology and leader in the field of breast cancer cell biology, and Alfed E. Chang, M.D., Professor of Surgery and Chief, Division of Surgical Oncology, Associate Director for Clinical Affairs of the University of Michigan Cancer Center and a established authority in the field of cancer immunotherapy. Included in the original grant was a subcontract with James M. Wilson, M.D., Ph.D., Director of the Institute of Human Gene Therapy, University of Pennsylvania and his colleague, Stephen L. Eck, M.D., Ph.D. More details regarding the investigators qualifications and budget justifications are found on pages 22-36 of the original research project proposal.

In the summer of 1995, Dr. Turka moved to the University of Pennsylvania but was retained as a consultant through a subcontract to the University of Pennsylvania. In September, 1996, Dr. Smith moved to Portland, OR where he serves as Chief, Clinical Research, Earle A. Chiles Research Institute, Providence Portland Medical Center. Because the Army Medical Research and Materiel Command considers that the research project grants are made to institutions and not individuals, a new Principal Investigator at the University of Michigan was named:

Laurence H. Baker, D.O.
Professor of Medicine and Associate Chief,
Division of Hematology/Oncology,
Department of Internal Medicine
University of Michigan School of Medicine
Director for Clinical Research and Deputy Director
University of Michigan Comprehensive Cancer Center

The University of Michigan has established a subcontract with the Earle A. Chiles Research Institute to allow Dr. Smith to continue to help coordinate the research grant especially with the performance of tasks 3 and 4 in the statement of work, i.e. to conduct a Phase I clinical trial of B7 transduced autologous breast cancer cells as a vaccine and to perform immunological monitoring studies on the patients participating in

the trial. The joint enrollment of patients at the University of Michigan and the Providence Medical Center will be advantageous because the patient population (i.e. breast cancer patients with an easily obtainable source of autologous tumor) is relatively rare. Patient accrual will be improved by having both centers involved. Because the start of the clinical trial has been delayed by difficulty in manufacturing the adenoviral vector, there is less time to conduct the study and it would be extremely difficult for a single institution to complete the trial within the remaining time on the grant. The Earle A. Chiles Research Institute, Portland Providence Medical Center was the first institution to conduct a gene therapy protocol in the state of Oregon. Currently, there are two active gene therapy studies at that institution, including a protocol for breast cancer patients, funded by the NIH, that is similar to the present Army grant. Their proposal differs because it uses an HLA-A2 matched, allogeneic human breast cancer cell line (MDA-MB-231) instead of using autologous tumor, but both are transducing the breast cancer cells with B7 (CD80) to make the tumor immunogenic. Therefore, Dr. Smith and his colleagues at the Earle A. Chiles Research Institute are completely capable and eminently qualified to conduct the clinical protocol and immunological monitoring as described in the statement of work. Dr. Smith will be in frequent contact with his collaborators and in addition, will make two trips per year to the University of Michigan for on site meetings. Additional details about the changes in the principal investigator and the subcontract with the Earle A. Chiles Research Institute can be found in the appended documents.

Task 1

Task 1 in the Statement of Work is to develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer. The work is being conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

> *Specific Aim #1. Selective isolation of human breast cancer cells from primary tumors and metastases using monoclonal antibody-conjugated magnetic beads.*
>
> **Rationale:** In order to isolate a highly pure population of human breast cancer cells that are suitable for infection with adenoviral expression vectors it is necessary to prepare a viable single cell suspension of cells from breast cancer specimens. In our previous studies with primary and metastatic human breast cancer specimens we have used an enzymatic dissociation procedure to prepare breast cancer cells for cell culture experiments. For cell culture applications, generation of cell suspensions that consist of multi-cell aggregates of breast cancer cells and normal cells is sufficient and even advantageous in some ways. For the experiments to be performed in the present studies, the cell suspensions obtained following enzymatic dissociation of breast tissue specimens will be treated further

to prepare single cell suspensions. Single cell suspensions prepared from these specimens will be exposed to a panel of monoclonal antibodies in order to separate normal cells from malignant cells. Finally, the isolated breast cancer cells will be infected with adenoviral expression vectors, containing either a reporter gene (LacZ) for developmental studies, or the B7 gene for gene therapy experiments.

a. Preparation of single cell suspensions of human breast cancer cells.

Solid tumor specimens, either primary tumors or solid metastatic nodules, will be minced with sterile scalpels until tissue pieces are approximately 1 mm^3. The minced tissues will be suspended in Medium 199 containing Worthington type III collagenase (Worthington Chemical Co., Freehold, NJ) at a concentration of 200 units per ml, and Dispase (Boehringer-Mannheim, Indianapolis, IN) at a concentration of 1 mg per ml. Twenty mls of enzyme solution are used per gram of tissue. The tissues are incubated overnight in a 37° water bath shaking at 65 rpm. The next day, the remaining tissue clumps are mechanically dissociated by repeated pipetting of the suspension. The cells are then washed three times by centrifugation at 250 x g and re-suspended in fresh Medium 199 after each wash. This enzymatic dissociation procedure results in a mixed suspension of single cells, small aggregates and large mammary organoids. The viability of the cells in this suspension is greater than 95%. To prepare a single cell suspension from the mixed aggregate population, the cells will be washed in Ca^{++}, Mg^{++}-free, Hanks balanced salt solution (CMF-Hanks BSS) and then incubated for 4 hours in CMF-Hanks BSS containing 10 mM EDTA, at 4° with gentle rocking. The cells will be mechanically dissociated every hour during the four hour period by repeated pipetting of the cell suspension. If necessary to maintain viability of the cells during this incubation, the CMF-Hanks-EDTA solution will be supplemented with 5% fetal bovine serum that had been treated with Chelex to remove divalent cations. After the four hour incubation, single cells are separated from any remaining cellular aggregates by filtration through Nitex mesh with a 20 um pore size.

Our preliminary data indicate that collagenase/Dispase dissociation of breast tissues does not adversely effect the integrity of cell surface molecules as these aggregates are quite reactive to antibodies directed against cell surface proteins. In generation of single cell suspensions, however, it is imperative that a method be used that does not alter the peptide epitope present on the surface of breast cancer cells. For this reason, we have chosen to use chelating agents that disrupt cell to cell interactions without degrading cell surface molecules to achieve the final single cell suspensions. It is necessary to obtain single cell suspension for the final cell purification procedures as the separation methods make use of antibodies that bind to epitopes expressed on beast cancer cells and not on normal mammary epithelial cells. If cell aggregates are used in the cell isolation

procedures and if aggregates contain both normal and neoplastic cells, then the purpose of using breast cancer specific antibodies would be defeated.

b. Isolation of breast cancer cells using antibody conjugated magnetic beads.

The basic strategy for isolating breast cancer cells involves the use of magnetic beads (Dynabeads, Dynal Inc. Great Neck, NY) that have been conjugated with anti-mouse IgG antibodies. Thus, the anti-mouse antibodies on the beads can be bound to mouse monoclonal antibodies directed against cell surface epitopes to prepare a reagent that specifically binds cells expressing the epitope. Following incubation of a cell suspension with antibody coated magnetic beads, the bound cells can be separated from non-bound cells by placing the tube in magnetic particle concentrators (MPC) designed to hold microfuge tubes. The beads and bound cells adhere tightly to the wall of the MPC and the non-bound cells are aspirated from the tube. The tube is then removed from the MPC, the cells re-suspended in medium and this washing procedure is repeated three to four times. With this method, we have separated mixed cell populations with greater than 99% efficiency using antibodies against the erbB-2 protein and antibodies against breast epithelial mucins. The cells isolated in this way have been seeded into culture and exhibit high viability as indicated by their ability to attach in culture and proliferate.

To coat magnetic beads with mouse monoclonal antibodies, 1×10^8 anti-mouse IgG Dynabeads are suspended in 1 ml of CMF-Hanks BSS and incubated with 1 μg of mouse monoclonal antibody with rocking at room temperature for two hours. Following the incubation, the beads are washed extensively with CMF-Hanks BSS by adhering the beads to the tube wall using the MPC, aspirating the medium, re-suspending the beads in 1 ml of fresh medium and incubating with rocking for 30 minutes. This washing procedure is repeated three times. After the last wash, the beads are suspended in CMF-Hanks BSS at a concentration of 10^8 beads per ml.

To isolate cells using antibody conjugated magnetic beads, 2×10^7 magnetic beads are added to a 1 ml aliquot of a cell suspension of 1×10^7 cells and incubated with rocking at room temperature for two hours. Next, the cells bound to the beads are washed three times to separate them from non-bound cells. If necessary, the beads can be removed from the purified cells either by trypsinization or by incubation with the peptide epitope that was used to generate the primary antibody. The cells isolated in this way can be used to initiate cell cultures of breast cancer cells or can be infected with adenoviral expression vectors. For the experiments to be carried out in this project, we will employ magnetic beads coated with three different antibodies. The first antibody, Sm-3, was generated against the core peptide of breast epithelial mucins. As discussed earlier, altered glycosylation of mucins that occurs in greater than 90% of breast cancer cells reveals the peptide epitope that is masked in normal cells by glycosylation. Thus,

the Sm-3 antibody coated beads will be the primary antibody for isolating human breast cancer cells from primary tumor specimens that contain both normal and neoplastic mammary epithelial cells. The Sm-3 antibody was obtained from Dr. Joy Burchell, Imperial Cancer Research Fund, London, UK. A second antibody, Mc-5, recognizes breast epithelial mucins expressed on virtually all breast cancer cells. This antibody also binds to normal mammary epithelial cells making it less useful for primary tumor specimens. However, magnetic beads coated with this antibody have been used in our laboratory to isolate breast cancer cells from metastatic lymph nodes and pleural effusion metastasis. This antibody was obtained from Dr. J. Peterson, Cancer Research Fund of Contra Costa, Walnut Creek, CA. Finally, a third antibody, Tab-254, binds to the extra-cellular domain of the erbB-2 protein. Magnetic beads conjugated with this antibody are useful in isolating breast cancer cells from primary or metastatic sites that overexpress the erbB-2 protein as a result of amplification of the c-erbB-2 (Her-2/neu) gene, which occurs in approximately 30% of breast cancer cases. A panel of Tab antibodies against the erbB-2 protein have been obtained from Dr. Beatrice Langton, Berlex Biosciences, Richmond, CA.

During the past year, Dr. Stephen Ethier's developed improved methods for the isolation and cultivation of primary human breast cancer cells in vitro. His laboratory has now isolated and characterized ten new human breast cancer cell lines. Four of these cell lines are from primary tumors, one was isolated from a metastatic lymph node, one is from a skin metastasis, one was from a recurrent chest wall lesion and the remaining cell lines are from pleural effusion metastasis or malignant ascites. The cell lines express the range of oncogene changes known to occur in human breast cancer including; erbB-2 amplification and overexpression, overexpression of epidermal growth factor receptor, amplification of the FGFR 1 and 2 genes, mutations in P53 and alterations in pRB protein expression. A summary of the molecular characteristics of all the human breast cancer cell lines that have been isolated and cultivated by Dr. Ethier's lab is found in Table 1 and more details can be found in references 3-7.

These lines represent the kind of range of human breast cancer cells that will need to be used in gene therapy studies. Furthermore, the isolation of these lines is indicative of the progress that his lab has made in developing methods and conditions for the routine isolation of human breast cancer cells. Thus, successful short term culture of human breast cancer cells is now possible for the majority of patient samples, and long-term cell culture of these breast cancer cells is possible with roughly 20% to 30% of specimens.

Dr. Stephen Ethier's laboratory has performed some experiments to purify breast cancer cells that were only marginally successful. They used the Sm-3 antibody, which is reported to be luminal cell specific, attached to magnetic beads to purify cells. The overall methodology worked well, but the antibody was not specific enough and many cancer cells were not purified using this approach. They are currently doing more experiments

with magnetic beads using antibodies to a cell surface marker called CALLA-1 and to Muc-1 and expect that these will be more useful in purifying breast cancer cells. They are also using a microgravity cell generator to selectively isolate breast cancer cells. The differential ability of breast cancer cells to survive and grow in suspension is the basis of this method and thus far they have found that a 6 to 10 day culture in suspension results in death of the majority of normal cells and the survival of the breast cancer cells. It is expected that in future experiments, the antibody-magnetic bead approaches will be combined with the microgravity cell generator to obtain nearly pure populations of breast cancer cells.

Task 2

Task 2 in the Statement of Work is to study and optimize the efficiency of and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA. The work is being conducted in the laboratory of Stephen P. Ethier, Ph.D. at the University of Michigan. The methods that were proposed in the original grant are described in detail below.

Specific Aim #2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

Rationale: Prior to the use of transduced breast cancer cells as immunotherapy the methodologies to transduce the maximum number of cells with an adenoviral vector and to verify that large numbers of cells express the vector-encoded human gene for at least several days must be developed. In this specific aim, we will perform a series of experiments aimed at optimizing methods for the infection of purified human breast cancer cells with adenoviral expression vectors and for optimizing the expression of a transgene within these vectors. Experiments will also be performed to determine the immunogenicity of human breast cancer cells that express the B7 gene following infection with appropriate adenoviral expression vectors.

a. Infection of purified human breast cancer cells with adenoviral expression vectors.

To perform the optimization experiments, an adenoviral vector containing a reporter gene (LacZ) as the transgene will be used. In preliminary studies with early-passage breast cancer cell lines developed in our laboratory, we have found that overnight exposure of these cells to these adenoviral vectors results in expression of the LacZ transgene in greater than 80% of the cells. To optimize infection of purified human breast cancer cells with the adeno-LacZ virus, aliquots of 10^6 human breast cancer cells, purified using methods described above, will be

incubated in suspension with adeno-LacZ virus for 24 hours with gentle agitation. Multiplicity's of infection ranging from 10^2 to 10^4 pfu's per cell will be tested in these experiments. Following infection, cells will be seeded into culture using media that we have developed for human breast cancer cell growth, and the cells will be assessed for LacZ activity at 24 hours, 3, 7, 10 and 14 days after infection. This experiment will be carried out with cells from at least 10 separate breast cancer patients. In these experiments, we will determine the optimum multiplicity of infection and the duration of the transgene expression in purified human breast cancer cells infected with adenoviral vectors immediately after their isolation.

b. Infection of purified human breast cancer cells with B7-adenoviral vectors.

Experiments will then be performed to transduce human breast cancer cells with the human B7 gene using adenoviral vectors developed by James Wilson and Steve Eck (see letter of consultancy). For these experiments the conditions shown to yield optimal transduction of the LacZ reported gene will be used for B7. Expression of B7 protein on the surface of the human breast cancer cells will be assessed by flow cytometry using CTLA4Ig or commercially available mouse anti-human B7 mAb. We will also verify that the cells are capable of supporting B7-mediated responses such as providing co-stimulatory signals (as assessed by proliferation and IL-2 gene expression) for autologous T lymphocytes activated with phorbol ester, bacterial superantigens, or PHA (methods as outlined under specific aim #4). Each of these is an accessory cell dependent stimulus, however we and others have shown that purified T cells can respond to these stimuli in the presence of B7-transfected CHO cells. Thus we will use B7-transfected CHO cells as a positive control in these studies. This also will allow us to compare the relative co-stimulatory abilities of $B7^+$ CHO cells and autologous $B7^+$ breast cancer cells. Since in the design of our phase I study (specific aim #3) the cells will be irradiated with 5000 cGy prior to injection into patients, we will also verify in these studies that the cells retain co-stimulatory capacity after irradiation at this dosage.

In previous reports, we have indicated that human breast cancer cells infected with an adenoviral expression vector containing that LacZ gene, express the transgene at very high frequencies (approximately 90%) and maintain expression of the transgene for several weeks following infection. More recently, Dr. Ethier's lab has performed experiments with an adenoviral expression vector containing the human B7-1 gene. This vector is essentially the same vector that will be used to prepare the autologous tumor cell vaccines for the clinical trial. The experiments performed thus far have made use of this vector (AdB7-1) and a series of human breast cancer lines that he has developed in his laboratory. There are a number of advantages of the cell lines he has developed. Since they were developed in his lab, they have all been tested in early passage, and thus are more representative of the breast cancer cell primary cultures that will eventually be used in the clinical trial. In addition, his lines come from a wide range of breast cancer

specimens, ranging from early stage primary tumors, to large inflammatory primary tumors to metastatic specimens and chest wall recurrences.

To do these experiments, cells from the human breast cancer cell lines were cultured to high density and then infected for 2 hours with the AdB7.1 vector at various multiplicity's of infection (MOI). After 48 hours, the cells were harvested from the dish using 10 mM EDTA, and incubated with the high affinity B7 binding protein CTLA4Ig. The cells were then incubated with a fluorescent secondary antibody, washed extensively and then scanned by flow cytometry.

The FACs scans shown in figure 1 illustrate the detection of B7.1 expression in the human breast cancer cell lines 48 hours after infection. Table 2 shows the data obtained from these scans and indicates the percent B7 positive cells following infection at two different MOIs. These results indicate that all of the human breast cancer cell lines studied were successfully infected and expressed the B7 protein on the cell surface. Although there was some variability from experiment to experiment, these experiments indicated that at MOIs of 10^4 particles per cell, the vast majority of breast cancer cells express high levels of the protein on the cell surface. At lower MOIs, most of the breast cancer cells did express the B7 protein, but the proportion of positive cells was lower.

These results clearly indicate that human breast cancer cells from many different patients and derived from both primary and metastatic sites are successfully infected with the AdB7.1 vector and express high levels of the protein on the cell surface. In addition, the expression of B7.1 protein following infection is independent of the growth rate of the breast cancer cell lines. Indeed, SUM-44 cells, which still have doubling times of approximately 200 hours, are as readily infected as SUM-149 cells, which grow much more rapidly. Experiments currently underway are aimed at extending these experiments to studies with cells derived directly from patient samples, and examining the influence of radiation exposure on the long-term expression of B7 protein in these cells.

Task 3

Task 3 in the Statement of Work is to conduct a Phase I clinical trial of B7 transfected breast cancer cells as a vaccine. Patients will be enrolled onto the clinical trial at the University of Michigan and at Portland Providence Medical Center. The detailed and complete phase I protocol was included in the original grant proposal. A summary of the methods that were proposed in the original grant are described in detail below.

> *Specific Aim #3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells as a vaccine to enhance the immune response to the tumor.*

Rationale: In order to test the safety of a genetically modified autologous tumor vaccine, a phase I clinical trial must be performed. Although other cancer patients have received genetically altered tumor cell vaccinations (transfected with cytokine genes), this trial would represent one of the first times a genetically altered tumor cell vaccine that contained the gene for B7 was given. Therefore, it is prudent to begin the testing in patients with advanced refractory breast cancer. This is not the optimal setting for a vaccine to work, since these patients will have a significant tumor burden and have received several chemotherapy regimens, both of which can significantly depress the immune system. Ideally, a vaccine should be utilized to treat patients with low tumor burdens such as is the case after primary definitive surgery and/or irradiation. Nevertheless, the initial toxicity and safety studies must always be performed in patients with advanced disease and limited life expectancy. We plan to administer an autologous irradiated B7-transfected breast cancer cell vaccine once to patients with advanced breast cancer and observe them closely for side effects, toxicities, any clinical anti-tumor responses, and changes in their immune response to the tumor. Cohorts of six patients each will receive one of 3 dose levels (numbers of transfected breast cancer cells transfected) in an escalating fashion as toxicity permits. Assuming toxicity is not severe, future clinical trials could consider a vaccination schedule with several planned vaccinations, as well as the addition of adjuvants (if necessary) or systemic immune stimulating agents such as IL-2 following the vaccine. Future studies could also incorporate the harvest of regional lymph nodes and expansion of TILs for adoptive immunotherapy. The details of the proposed clinical trial are described in the model protocol that is in Appendix F. The key features will be delineated below. We recognize that certain technical details are still in the developmental phase. These details will be incorporated into the final version of the protocol prior to submission to the IRB, RAC and FDA. We also wish to emphasize that the appropriate animal toxicity studies will be performed prior to the initiation of the clinical trial by our collaborators Drs. Wilson and Eck (at the University of Pennsylvania) using the type of B7-expression vectors that would be used in our human studies. These toxicity experiments will include intravenous injection of the B7 vector, as well as deliberate transduction of a variety of non-malignant cells such as hepatocytes, fibroblasts and keratinocytes. The studies will assess the animals for adverse effects including the induction of unwanted autoimmune responses.

Phase I Study Objectives.

1. To determine the toxicity of subcutaneously administered irradiated autologous breast cancer cells that have been transfected with the human gene for B7 in patients with advanced or metastatic breast cancer.
2. To determine the maximum number of transfected breast cancer cells that can be safely given to these patients.
3. To determine if the vaccination results in an immune response and to characterize that immune response.

4. To observe patients for any antitumor responses.

Eligibility Requirements.

Patients must have advanced breast cancer that has failed to respond to at least two standard chemotherapy regimens used in the metastatic setting and who are considered unlikely to benefit from further salvage chemotherapy regimens or hormonal regimens. They must also have a source of autologous tumor that can be easily harvested. This includes patients with subcutaneous or cutaneous metastases, patients with easily excisable lymph nodes containing metastatic tumor, and patients with malignant pleural effusions or ascites. Patients must have a good performance status and a life expectancy of at least three months. Patients must be at least 18 years old. There is no exclusion for sex or ethnic background. Patients must have evaluable or measurable disease in addition to the disease that will be surgically removed for the purposes of formulating the autologous vaccine. Adequate baseline organ function will be required. In addition, patients must not be anergic to standard recall antigens. Patients may not have received prior antitumor vaccines or immunotherapy. Patients will be excluded if they have any autoimmune diseases, evidence of HIV infection or AIDS, active infection, bleeding, pregnancy, or lactation, or any significant uncontrolled medical or pyschiatric illness. Patients who require corticosteroids or anticoagulation are ineligible.

Study Design.

Patients will undergo surgical removal of metastatic disease under local anesthesia in order to provide autologous tumor cells that can be transfected with the human B7 gene. A section of the removed tumor will be sent to surgical pathology for pathologic diagnosis. The remainder of the specimen will go to the laboratory to prepare B7-transfected autologous tumor, for immunologic assays, and for cryopreservation. (For details on the purification and transfection of the breast cancer cells see the technical methods section for specific aim 1 and 2). After the autologous breast cancer cells have been transfected with B7 they will be irradiated with 5000 cGy, a dose of radiation that renders them nontumorigenic but allows them to remain metabolically active. They will then be injected intradermally into the thigh approximately 10 cm below the inguinal lymph nodes and the injection site will be marked with an ink tatoo for future biopsy. The injections will be administered in the Clinical Research Center, University of Michigan Hospital and the patients will remain in the hospital over night. Cohorts of six patients each will be treated with escalating doses of autologous irradiated B7 transfected breast cancer cells according to the following scheme: a): 10^6 cells, b): 10^7 cells, c): 10^8 cells (for technical reasons, 10^8 cells is the likely maximum number of cells that could be obtained from these patients). Individual patients will receive one dose level of cells (i.e. there is no intrapatient dose escalation). Only one vaccination is planned for each patient unless the patient demonstrates clinical benefit from the treatment, whereupon the patient can receive

additional monthly vaccinations as long as such benefit persists. Each patient will be observed for at least three weeks at a given level of cell injection before the patients are permitted to enroll on the next higher dose level of cells. If one or fewer patients experience dose-limiting toxicity at a given number of cells injected, escalation will be permitted to continue to the next level. If two or more patients sustain dose-limiting toxicity, then that level of cells will be determined as the dose-limiting number of cells and the dose level of cells below that will be defined as the maximum tolerable dose of cells to be injected. It is possible that at the maximum dose of B7-transfected cells dose-limiting toxicity will not be observed.

Once escalation is completed, a separate cohort of six patients will be treated with both B7-transfected autologous irradiated breast cancer cells and vector only-transfected autologous irradiated tumor cells. One injection will be placed in the left thigh and one in the right thigh at the same time. The purpose of treating this cohort of patients is to compare the immunologic response at the vaccination site and in the draining lymph nodes from one leg to the other. This will help determine if B7 transfection enhances the immune response above that which is seen with transfection of vector alone.

Study endpoints.

TOXICITY - Patients will be closely followed and observed for the development of any clinical side effects from the treatment. Toxicity will be graded according to the Cancer Treatment Evaluation Program toxicity scale. The major toxicity that is anticipated is local redness, swelling, pain, and increased warmth at the injection site. Patients will be monitored for the development of clinical symptoms suggesting autoimmune disease or allergic reactions. Changes in laboratory parameters (complete blood count, chemistry panel, coagulation studies, urinalysis, as well as tests for the development of autoimmune disease [ANA, RF, CH50, anti-DNA abs, T4, TSH]) will be assessed two and four weeks after vaccination and thereafter once/month. In addition, sera and peripheral blood mononuclear cells will be obtained for archival purposes according to the current safety monitoring guidelines by the Center for Biologics Evaluation and Research (presently, once/month on treatment and every three months thereafter).

IMMUNE RESPONSE - A biopsy of the vaccination site along with surgical removal of one to three draining inguinal lymph nodes will be performed two weeks after the vaccination. Peripheral blood will be obtained at two weeks and 4 weeks and then once/month. DTH skin testing will be performed monthly. The details of the immunologic monitoring are extensively described in the methods section for specific aim #4.

ANTITUMOR RESPONSE - Four weeks after vaccination, the patients will undergo reevaluation to determine if their disease has responded or progressed using standard response criteria. Patients whose disease has not worsened or has regressed (even if it does not meet the criteria for partial regression) will be eligible to receive additional cycles of treatment using the autologous irradiated B7-

transfected cancer cells providing that they experienced no severe toxicity with the first vaccination. Patients may continue this treatment until they have evidence of progressive disease.

Initiation of work on Task 3 is dependent on the development of a FDA-approved, replication-defective, recombinant adenovirus bearing a human B7 cDNA (Ad.hB7) for use in human clinical trials. Our subcontractors at the University of Pennsylvania, Dr. James Wilson and Dr. Stephen Eck, have made significant progress toward this goal and anticipate that breast cancer patients will start accruing onto the Phase I trial in 1997. The following data summarizes their preclinical experience.

Ad.hB7-1:
in vitro gene transfer - Ad.hB7-1 transduces a variety of mammary and melanoma cell lines in vitro to express hB7-1. Using antibody staining and flow cytometry, at an MOI of 10, about 32% of WM9 human melanoma cells express hB7-1 three days after transduction; at an MOI of 100, nearly 100% of WM9 cells express hB7-1 three days after transduction..

in vivo gene transfer - Ad.hB7-1 was injected into WM9 human melanoma tumors established in the flank of SCID mice. Flow cytometric analysis of single cell suspensions made from the injected tumors revealed that 26%, 19% and 32% of tumor cells expressed hB7-1 at 7, 14 and 21 days after injection, sequentially.

Cells transduced with Ad.hB7-1 deliver a co-stimulatory signal to human T cells - Ad.hB7-1-transduced or Ad.lacZ-transduced WM9 human melanoma cells (89%+ for hB7-1 and 94%+ for lacZ, respectively, at three days; WM9 cells constitutively express MHC class I and II antigens) were treated with mitomycin C and cocultured with purified, allogeneic human peripheral blood T cells. T cell proliferation stimulation indices calculated on the basis of ^{3}H-thymidine incorporation by the T cells indicated that there was a 33-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by Ad.lacZ-transduced WM9 cell and a 9-fold greater stimulation of proliferation by Ad.hB7-1-transduced WM9 cells than by untransduced WM9 cells. Similar results were obtained with Ad.hB7-1-transduced chinese hamster ovary (CHO) cells and WM793 human melanoma cells.

Ad.mB7-1:

in vitro gene transfer - Ad.mB7-1 transduces murine mammary and melanoma cell lines in vitro to express mB7-1 by flow cytometry. At an MOI of 1000, nearly 100% of K1735 murine melanoma cells express mB7-1 three days after transduction.

in vivo gene transfer - Ad.mB7-1 injected into subcutaneous K1735 tumors established in immunocompetent C3H/HeN mice resulted in tumor cell expression of mB7-1 determined

by flow cytometry and immunohistochemical staining on days 3 and 7 but not on day 15. Extinction of expression is presumably due to immunological elimination of cells expressing adenovirus vector antigens, as has been described before.

Efficacy of mB7-1 delivered by Ad.mB7-1 in inducing tumor rejection - K1735 murine melanoma cells were transduced with Ad.mB7-1 or Ad.lacZ in vitro at an MOI of 1000. After three days, 10^6 transduced or parental K1735 cells were injected into C3H/HeN mice. By 8 weeks, all 10 mice injected with parental K1735 cells developed progressive tumors, while 7/10 and 0/10 mice injected with Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors. Mice injected with parental K1735 cells developed tumors at a median of 22 days, while mice injected with Ad.lacZ-transduced cells developed tumors at a median of 36 days. When surviving mice were rechallenged with parental K1735 cells 8 weeks after initial challenge, 3/3 and 3/10 of the mice that had survived their challenge of Ad.lacZ- and Ad.mB7-1-transduced cells, respectively, developed progressive tumors (all 10 naive mice injected at the time of rechallenge developed progressive tumors). The results obtained with Ad.mB7-1-transduced K1735 cells are comparable to those achieved using retrovirus-transduced, B7-1+ K1735 cells (including the relatively low frequency of protective immunity at 8-12 weeks in mice that previously rejected B7-1+ K1735 cells). Efforts to assess the efficacy of in vivo Ad.mB7-1-transduced K1735 tumor cells (Ad.mB7-1 injection intratumorally) have been unsuccessful. In part, this may be due to the fact that treatment cannot begin until the subcutaneous tumors reach a size that can be injected - about 3-4 mm in diameter. At this point, the rate of unperturbed K1735 tumor growth is such that C3H/HeN mice live a median of 8-9 additional days before they die of tumor or require euthanasia which may be insufficient time for effective host immunization and tumor control.

Ad.mB7-1 efficiently transduces SCK mammary carcinoma cells in vitro to express mB7-1. Injection of 2.5×10^4 live Ad.lacZ- or Ad.mB7-1-transduced SCK cells into A/J mice resulted in 9/10 mice in both groups developing tumors, while 6/10 mice given retrovirus-transduced mB7-1+ SCK cells developed tumors. An adverse effect on outcome of adenovirus transduction itself was excluded by the fact that a similar fraction (6/10) of mice developed tumors that had been given retrovirus-transduced mB7-1+ SCK cells supertransduced with Ad.lacZ. A potential explanation for the lack of protection by Ad.mB7-1-transduced SCK cells may come from the constraints of working with this aggressive tumor model. We routinely inject 2.5×10^4 live SCK cells after which tumors usually appear in 6-8 days and the mice are dead in 13-15 days. When we introduce 2.5×10^4 live SCK cells made B7-1+ by stable retrovirus transduction, all progeny SCK cells express B7-1, the number of B7-1+ tumor cells expands and the effective immunization dose increases. In contrast, when we introduce 2.5×10^4 live SCK cells made B7-1+ by Ad.mB7-1 transduction, assuming one viral genome/transduced cell, only half of the progeny of B7-1+ SCK cells will express B7-1, the number of B7-1+ tumor cells never increases above 2.5×10^4 and the effective immunization dose remains constant and low. Injecting more live SCK cells (e.g. 10^5 or 10^6 cells) is not a solution because these larger

inocula accelerate an already rapid disease course (death in 10-12 days) which we believe is inadequate to permit tumor immunization and immunological tumor rejection. Attempts to demonstrate the enhanced immunogenicity of Ad.mB7-1-transduced SCK cells by vaccination with 10^6 irradiated cells also have not succeeded, in part due to the enhanced immunogenicity of irradiated Ad.lacZ-transduced SCK tumor cells which may be preventing us from seeing an added benefit from vaccinating with mB7-1+, Ad-transduced cells.

The third round of plaque purification will be completed in the middle of May, 1997 at the Institute for Human Gene Therapy University of Pennsylvania. The FDA has indicated that the only toxicity study that will be necessary will be a study of Ad.mB7-1 in mice. It is anticipated that these studies will be completed by the end of August, 1997. The IND will be prepared and submitted by the Institute for Human Gene Therapy during August/September of 1997 and FDA approval is hoped for by October, 1997 and patient accrual onto the clinical trial by November or December, 1997.

Task 4

Task 4 is to perform in vitro and in vivo immunologic monitoring studies on patients enrolled on the clinical trial to assess the development of an anti-tumor immune response. Work on task 4 will begin as soon as patients begin treatment on the clinical trial estimated to start in the last quarter of 1997.

Additional animal experiments

Based on the recommendation of the group that reviewed the original grant, an animal model of breast cancer was developed to test the gene therapy proposed in the grant, before beginning the clinical trial in patients.

Dr. Fred Chang's laboratory at the University of Michigan studied a mammary carcinoma, MT-7 in Balb/c mice. The attached manuscript provides additional details and has been accepted for publication in *Cancer Gene Therapy*. MT-7 is a cultured tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the Balb/c host. A subline, MT-901, was derived from an early in vitro passage of cultured MT-7 tumor inoculated subcutaneously. MT-901 cells were determined to be weakly immunogenic in traditional immunization and challenge experiments. MT-901 cells that were genetically modified to express the co-stimulatory molecule B7-1 failed to generate tumors in two out of five mice that were inoculated subcutaneously whereas five out of five mice had tumor growth when inoculated with the wild-type MT-901 tumor cells. MT-901 cells that were genetically modified to secrete GM-CSF also grew less well than wild-type MT-901 with no tumor growth in two out of five mice inoculated with a low GM-CSF secreting clone.

In immunization and challenge experiments neither genetic modification resulted in superior protection against a subsequent tumor challenge compared to wild-type tumor alone. In separate experiments, MT-901 cells that were genetically modified to secrete IL-12, initially grew, but then were rejected in all five mice that were inoculated. However, subsequent challenge of these mice with wild-type tumor cells resulted in tumor growth in all the animals.

The genetically modified MT-901 tumor cells were then tested for their ability to sensitize tumor draining lymph node cells (TDLN) for adoptive immunotherapy. The TDLN were harvested nine days after the inoculation of tumor cells and they were activated and expanded with anti-CD3 plus IL-2 in vitro and adoptively transferred into mice bearing three day established MT-901 pulmonary metastases. The B7-1 expressing clone induced pre-effector cells better than wild-type tumor in one of two experiments. The low GM-CSF secreting clone was no different than wild-type tumor, but the high GM-CSF secreting clone was significantly better than wild-type tumor in the induction of tumor reactive TDLN. In a similar but separate experiment, the IL-12 transfected clone failed to elicit pre-effector TDLN cells differently from wild-type tumor.

Subsequent experiments designed to determine the type of effector cell that mediated tumor regression with the successful GM-CSF secreting clone indicated that $CD4^+$ cells mediate the effect. The CD4 mediated cytotoxicity appears to be related to *fas* ligation because the addition of *fas* fusion protein inhibited the in vitro cytotoxicity of these $CD4^+$ cells.

Dr. Chang's lab is currently exploring alternative approaches that may take advantage of B7-1 transgene expression, for example, by combining it with the co-expression of transgenes encoding for the cytokines GM-CSF or IL-12.

Additional experiments

Because of the delay involved in obtaining the adenoviral vector from our subcontractor, Dr. Ethier's lab conducted experiments to test the suitability of other vectors for possible use in the clinical trial. Lipofection with a plasmid vector and delivery by a gene gun failed to get the LacZ gene into more than a small percentage of cultured human breast cancer cell lines and was therefore abandoned as a possible alternative strategy.

III. Conclusions

The progress reported in this summary of year two of this grant indicates that the major objectives of Task 1 and Task 2 of the Statement of Work have been achieved, paving the way for the clinical trial that will begin in 1997. Dr. Ethier's laboratory has clearly demonstrated the feasibility of short-term culture of human breast cancer cells necessary to perform a vaccination trial where autologous tumor cells are transduced with an adenoviral vector ex vivo. Dr. Ethier's laboratory has also documented the success of the adenoviral expression vector, Ad.hB7, in both breast cancer cell lines and primary cultures of human breast cancer cells with more than 90% of the cells expressing B7-1 on their surface for at least one month. These accomplishments demonstrate that the experimental approach that will be utilized in the clinical trial, i.e. ex vivo incubation of autologous breast cancer cells with the human B7-1 adenoviral expression vector, Ad.hB7, is technically feasible and that these transduced cells should express B7-1 sufficiently long in vivo for vaccination purposes.

Experiments conducted in two different mouse mammary carcinoma models support the hypothesis that B-7 expression enhances the immune recognition of tumor cells. Both models also point to additional cytokines (i.e. IL-12 or GM-CSF) that may improve the immune mediated regression of tumors in combination with B7-1 expression and will be studied further in the next year. After the safety of B7-1 transduced tumor cells is demonstrated in patients, the addition of these cytokines can be contemplated.

IV. References

1. Aruga E, Aruga A, Arca MJ, Smith, JW, Chang AE. Immunotherapy of Murine Breast Cancer by Lymph node cells primed by tumor modified to secrete GM-CSF. Cancer Gene Therapy, 2:311, 1995. Abstract.

2. Aruga, A. Aruga, M. Arca, W. Lee, NS. Yang, J.W. Smith, A. Chang. Immune Responsiveness to a Murine Mammary Carcinoma Modified to Express B7-1, IL-12 or GM-CSF. (in press, *Cancer Gene Therapy*), 1996.

3. Ethier, S.P., Mahacek, M.L., Gullick, W.J., Frank, T.S. and Weber, B.L. Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sties using selective media. Cancer Res. 53: 627-635, 1993.

4. Mahacek, M. L., Beer, D., Frank, T.S. and Ethier S.P. Finite proliferative lifespan in vitro of a human breast cancer cell strain isolated from a metastatic lymph node. Breast Cancer Res. and Treat. 28: 267-276, 1993.

5. Ethier, S. P. Human breast cancer cell lines as models of growth regulation and disease progression. Journal of Mammary Gland Biology and Neoplasia 1: 111-121, 1996.

6. Ethier, S. P., Kokeny, K. E., Ridings, J. W., and Dilts, C. A. ERB-B family receptor expression and growth regulation in a newly isolated human breast cancer cell line. Cancer Res. 56: 899-907, 1996.

7. Sartor, C. I., Dziubinski, M. L., Yu, C-L., Jove, R. and Ethier, S. P. The role of EGF receptor and STAT-3 activation in autonomous proliferation of SUM-102PT human breast cancer cells. Cancer Res. 57: 978-987, 1997.

Table 1.

Molecular Characteristics of "SUM" HBC Cell Lines

Cell Line	Oncogene Amp.*	EGFR	erbB-2	erbB-3	erbB-4	p53 (IHC)
SUM-44PE	FGFR-1	–	+	+	+	+ (c)
SUM-52PE	FGFR-1 & 2	–	+	+	+	+ (c)
SUM-16LN	EGFR	++++	NE	NE	NE	NE
SUM-102PT	none	+++	+	+	–	–
SUM-149PT	none	+++	+	+	–	+ (n)
SUM-159PT	none	+	+	–	+	+ (n)
SUM-1315MO2	NE	+	+	–	+	+ (n)
SUM-185PE	erbB-2 (?)	+	+++	NE	NE	NE
SUM-190PT	erbB-2 (?)	+	+++	NE	NE	+ (n)
SUM-206CWN	NE	+	++	NE	NE	+ (n)
SUM-224PE	NE	NE	NE	NE	NE	NE
SUM-225CWN	NE	NE	NE	NE	NE	NE
SUM-229PE	NE	NE	NE	NE	NE	NE

*Oncogenes examined by Southern blot: erbB-2, c-myc, Prod-1, FGFR-1, 2, 4, EGFR
NE - not examined

Note that in the table, cell lines designated PT are from primary tumors, PE are from pleural effusions, LN are from metastatic lymph nodes, and CWN are from chest wall nodules.

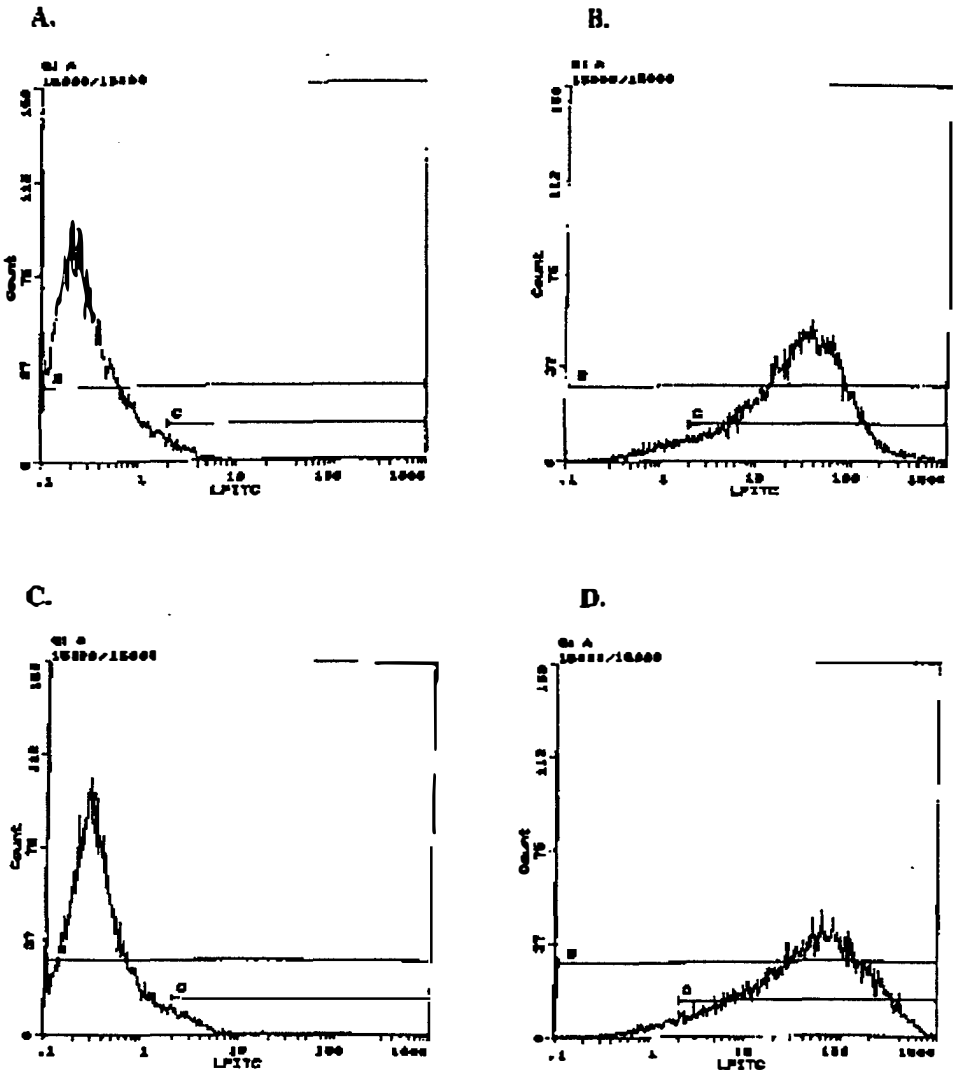
Table 2

B7 Expression in Human Breast Cancer Cell Lines

Cell Line	Multiplicity of infection (pfu/cell) 0	10^4	10^3
Sum-102	7.9	98.4	ND
	3.3	84.7	ND
Sum-149	4.3	92.8	ND
	5.1	75.6	38.9
Sum-159	3.0	92.9	ND
	12.4	97.9	ND
Sum-44	8.7	96.9	ND
	2.2	58.3	34.0
Sum-52	6.6	79.2	75.1

[a] percent B7-1 positive cells by flow cytometry

Figure 1.



FACS analysis of B7.1 expression in two human breast cancer cell lines. Panels A and B show data from SUM-149 cells and panels C and D show data from SUM-159. Left hand panels show background fluorescence of control cells, and panels B and D show B7.1 specific fluorescence of AdB7.1 infected cells.

VI Appendices

Original research project proposal. Appendix 1

Letter to Danny Laspe. Appendix 2

Subcontract with Earle A. Chiles Research Institute. Appendix 3

Attached manuscript (in press, *Cancer Gene Therapy*): Immune Responsiveness to a Murine Mammary Carcinoma Modified to Express B7-1, IL-12 or GM-CSF. E. Aruga, A. Aruga, M. Arca, W. Lee, N. Yang, J.W. Smith, A. Chang. (1996) Appendix 4

DRDA Proposal No. _____

THE UNIVERSITY OF MICHIGAN
PROPOSAL APPROVAL FORM

(see back of form for instructions)

PROPOSAL TYPE

___ Instructional X Research ___ Other Sponsored Activity

Prepared by Amy Laura 936-5281 (Telephone)

Date Prepared 11-17-93

If Continuation, Account No. _____

1. SUBMITTED TO United States Army (Direct Sponsor) — Ft Detrick, Frederick, MD 21702-5012 (Direct Sponsor Address (see instructions))

2. _____ (Prime Sponsor (if any)) — _____ (Direct Sponsor Address (see instructions))

3. PROJECT TITLE Gene Therapy of Human Breast Cancer

4. MAJOR FIELDS OF STUDY TO WHICH PROJECT IS RELATED (list three keywords *not* contained in the Project Title)

Immunotherapy | gene therapy | costimulation of T Cells

5. a. John W. Smith II, MD (PROJECT DIRECTOR) — Int Med: Hem/Onc (Department/Unit) — 2400 (Org. Code) — 6-5281 (Telephone)

b. Laurence Turka, MD (Participating Investigator) — Int Med: Nephrology (Department/Unit) — 2430 (Org. Code) — 6-4812 (Telephone)

Stephen Ethier, Ph.D (Participating Investigator) — Radiation Oncology (Department/Unit) — 3015 (Org. Code) — 6-7380 (Telephone)

Alfred Chang, MD (Participating Investigator) — Surgery (Department/Unit) — 3025 (Org. Code) — 6-4392 (Telephone)

Barbara Weber, MD — Int Med: Hem/Onc — 2400 — 7-3421

List additional participants under Notes (p

[redacted] PII Redacted

gwil

6. PROPOSED PROJECT TIME PERIOD 10/1/94 (Start Date) — 9/30/98 (End Date)

7. PROPOSED PROJECT PERIOD BUDGET

	U-M Sources	Sponsor	Total
Total Direct Costs		551,038	
Indirect Costs		247,252	
TOTAL		798,290	

Indirect Cost Rate 52 %

8. DETAILS OF U-M SOURCES (Cost Sharing)

Line Item	Amount	Account Number	Org. Code	Approved by

Notes:

The undersigned certify, to the best of their knowledge and belief, that no federal appropriated funds have been or will be paid to influence or attempt to influence the granting of this award. We certify the proposed work is consistent with University unit objectives and all faculty involved in the proposal have agreed to participate. We accept the obligations and commitments described, and agree to perform the work in accordance with University and sponsor policies.

John W. Smith II, M.D. — Requested by: Project Director

Susan D. Osius Mng. — Fiscal/Legal Review

Gerald P. Weil, Rad Onc — Approved by: Dept or Unit Head

Approved for DRDA by: Project Representative

Approved for School/College by: Dean

Approved for the University

DRDA Proposal No. _____

9. Does the proposal activity involve:
(A "yes" or "no" response *is* required for each item in this section)

Use of human subjects	___ No	X Yes	If yes, date of committee approval PENDING
Use of vertebrate animals	X No	___ Yes	If yes, date of committee approval
Use of radioactive materials	X No	___ Yes	If yes, date of committee approval
Carcinogen comment	X No	___ Yes	If yes, specify
Recombinant DNA	___ No	X Yes	If yes, specify Plasmid DNA
Biological hazards	___ No	X Yes	If yes, specify Recombinant DNA
Propr etary materials	X No	___ Yes	If yes, attach sheet detailing proprietary materials involved
Classified research	X No	___ Yes	If yes, contact DRDA project representative for appropriate reviews and approval
Other restrictions on openness of research	X No	___ Yes	If yes, contact DRDA project representative for appropriate reviews and approval
Is a subcontract contemplated?	X No	___ Yes	If yes, contact Contract Administration
Potential conflict of interest	X No	___ Yes	See reverse side for explanation
Work off University property	X No	___ Yes	If yes, identify location of research
Study of another country	X No	___ Yes	If yes, name of country

10. Number of participating students Fellow 0 Graduate _____ Undergraduate _____

11. Have U-M funds been used as seed money or pilot project support for th s proposal?
X No _____ Yes If yes, provide Source _____ Amount $ _____

12. University space to be used

Room Number	Building	Bldg. Zip	Approved by Dean/Director
Reception C	Taub an		[signature]
1560	MSRB II	0676	[signature]
B2C490	University Hospital	0010	[signature]
2920K	Taubman	0031	[signature]
1518	MSRBI		[signature]

(List additional space under NOTES)

13. If insufficient space is available, indicate square feet required _____

Source of space _____ Approved by _____
VP for Research

If renovation is required, indicate dollar cost _____

U-M account number to be charged _____ Approved by _____

14. Is additional office equipment required for this project? X No _____ Yes

Is it provided for in the proposal? _____ No _____ Yes

If not, attach list and indicate University unit to prov de _____ _____
Approved

15. NOTES:

THE UNIVERSITY OF MICHIGAN
DIVISION OF RESEARCH DEVELOPMENT AND ADMINISTRATION

3003 South State Street
Ann Arbor, MI 48109-1274

November 29, 1993

Breast Cancer Research
U. S. Army Medical Research
and Development Command
Fort Detrick, Frederick, MD 21702-5012

Attention: SGRD-PLF
301-619-7631

Subject: The Regents of the University of Michigan Proposal No. DRDA 941453
Entitled: "Gene Therapy of Human Breast Cancer"

Enclosure: Original plus 20

The Regents of the University of Michigan submit for your review the subject proposal as a research program in the Department of Internal Medicine.

The project will be under the direction of John W. Smith II, M.D. The proposal is for a 48 month period at an estimated cost of $798,290.

For questions of a technical nature, please contact the project director at (313) 936-5281. For questions of an administrative nature, please contact me at (313) 763-6438.

Sincerely,

Neil D. Gerl, Ph.D.
Project Representative

NDG/dsb

cc: John W Smith, II
Amy Laura
941453

APPENDIX A

Research Proposal
Cover Page

Date: 11-17-93

1. **Name and Address of Offeror (Institution)**
   Regents of University of Michigan
   3003 S. State Street, Ann Arbor, Michigan 48109-1274
2. **Type of organization (check all that apply):**
   ( ) Large Business (X) Nonprofit ( ) Small Business
   ( ) Woman-Owned ( ) Foreign ( ) Disadvantaged Business
   (X) Educational Institution ( ) HBCU ( ) MI
   (X) State Government ( ) Federal Government ( ) Other
3. Title: Gene Therapy of Human Breast Cancer

4. (Career Development Applicants) Is a New Investigator proposal being submitted? _____ Title:

5. Submitter's Log No. 941453 6. USAMRDC Log No.*
7. Total Funding Requested 798,290 8. Requested Start Date: 10-1-94
9. Duration: 4 years

	Name and Department	Phone
10. Principal Investigator(s):		
Primary:	John W. Smith II, M.D.	313-936-5281
Alternate:	Laurence Turka, M.D.	313-936-4812
11. Administrative Representative Authorized to Conduct Negotiations:		
Primary:	Neil D. Gerl	313-763-6438
Alternate:	Dave Plawchan	313-764-7237

12. Authorized Representative:

Alan Walter Steiss
Typed Name

Signature

Director, DRDA
Title

November 29, 1993
Date Signed

*To be entered by USAMRDC

NOTHING ON THIS PAGE IS PROPRIETARY INFORMATION

APPENDIX B

Proposal Checklist

1. Contracting Organization: Regents of the University of Michigan

2. Address: 3003 S. State, RM 1058
Ann Arbor, MI 48109-1274

3. Principal Investigator: John W. Smith II, M.D.

4. Total Funding Requested: 798,290

5. This proposal requests funds for (check only one):

____Training and Recruitment ____Infrastructure Enhancement
_X_Research Project

6. For Training and Recruitment proposals, please select the category which applies (check one)

____Predoctoral Training ____Predoctoral Fellowships
____Special Sabbatical ____Postdoctoral Fellowships
____Career Development

7. For Infrastructure Enhancement proposals; please select the category which applies (check one).

____Enhancement of Existing Cancer Registries

____Registries of High-Risk Women

____DNA Resources (Clones, DNA Markers)

____Transgenic Mouse Husbandry

____Banks of Tumor Samples, Breast Tissues, and Cell Lines

____Information Systems

____Other Innovative Shared Resources (identify)

_____ _____

NOTHING ON THIS PAGE IS PROPRIETARY INFORMATION

11. For all proposals, please indicate which of the following apply:

_X_ Human Subjects, Clinical Trials

_X_ Human Subjects, Anatomical Samples

____ Laboratory Animals (identify)

_____ _____

_____ _____

_____ _____

____ Good Manufacturing Practices

_X_ Recombinant DNA

_X_ Hazardous Materials

____ Good Laboratory Practices

____ Investigational Drugs

____ Radioactive Materials

12. Optional Response:

Principal Investigator: Male (X) Female ( )

Minority: Yes ( ) No (X) Ethnic Origin: _____

Number of years of professionial/postdoctoral experience: 12

To be signed by:

Official of the Institution — November 29, 1993 — Date

John W. Smith II, M.D.
Principal Investigator — November 26, 1993 — Date

NOTHING ON THIS PAGE IS PROPRIETARY INFORMATION

APPENDIX C

Title Page

Title of Study (120 characters maximum)

Gene Therapy of Human Breast Cancer

Keywords (6-8 words)

Immunotherapy, gene therapy, vaccination, costimulation of T cells

Abstract

Two paragraphs - 250 words maximum.
First paragraph - Technical objective.
Second paragraph - Approach: Experiments to be performed; methods to be used.

The objectives of this proposal are to: 1) develop methods to isolate highly purified populations of breast cancer cells obtained from patients with advanced, refractory breast cancer 2) transduce these cells with the human B7 gene using adenoviral vectors 3) determine the efficiency and longevity of this transduction 4) conduct a Phase I clinical trial assessing the toxicity of these autologous irradiated B7-transduced breast cancer cells as a vaccine to enhance the immunological recognition of the tumor by costimulating T cells and 5) perform immunologic monitoring studies on these patients to assess development of an antitumor immune response.

Patients with advanced, refractory breast cancer with accessible metastases will undergo a minor procedure to harvest autologous tumor cells. These specimens will be enzymatically digested, then mechanically dissociated and filtered to prepare single cell suspensions which will be exposed to a panel of monoclonal antibodies in order to separate malignant cells. The isolated breast cancer cells will be infected with an adenoviral expression vector containing the human B7 gene. Experiments will be performed to determine the optimum multiplicity of infection and the duration of transgene expression. Cohorts of patients will be vaccinated with escalating numbers of autologous irradiated B7-transfected breast cancer cells and closely observed for toxicity. Biopsies of the vaccination site and draining lymph nodes as well as samples of the peripheral blood will be obtained and tested for the development of T cell immunity and humoral immunity against autologous B7-transfected tumor and vector-alone transfected tumor.

Proposal Relevance

This page will be used for a separate statement, one page or less in length, to make a case that this project is relevant to one or more critical issues in the prevention, detection, diagnosis, or treatment of breast cancer. This sheet (Appendix D) will follow the title/abstract page.

The annual number of new breast cancer cases and the number of deaths due to breast cancer continues to rise. Despite advances in treatment such as the development of effective chemotherapy and hormonal therapy regimens, there has been essentially no change in overall breast cancer mortality for the last 60 years. Although research will likely lead to the discovery of additional antineoplastic chemotherapeutic or hormonal agents, clearly, there is room for improvement in the treatment of breast cancer.

Another completely different modality of treatment that has shown some promise in the treatment of other cancers (e.g. melanoma, renal cell carcinoma and lymphoma) is immunotherapy. Up to now, immunotherapy has not been utilized to a great extent in the treatment of breast cancer. Vaccine therapy has been particularly limited in part because breast cancer cells have been extremely difficult to isolate and grow in culture. In addition, vaccine therapy has not been well utilized because of our limited understanding of how to induce a host anti-tumor response. Recently, it has been shown that T cell require two signals for optimal responses. The first is engagement by antigen, and the second is a co-stimulatory signal which can be provided through interaction of the T cell surface molecule CD28 with its natural ligand B7. Further studies have shown that transfection of tumor cells with B7 is sufficient to provide the costimulatory signals needed for complete T cell activation and tumor rejction. The presentation of tumor antigens in the absence of the costimulatory signal may lead to tolerance and could explain why many tumors are not rejected by their host.

This proposal is designed to capitalize on these recent developments in our understanding of the immune system in an attempt to harness its tremendous power and manipulate it to reject the growing tumor in the host. This proposal also will take advantage of the recent successes in our ability to isolate and maintain breast cancer cells in culture and to transduce them with a gene whose will make these cells more immunogenic. We feel the research conducted in this study will be a crucial first step on the path to improved treatment of breast cancer.

APPENDIX E

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I. Background and Significance

1. Overview.

The American Cancer Society estimates that there will be 182,000 new cases of female breast cancer and 46,000 deaths from breast cancer in 1993 (1). Between 1980 and 1987 the incidence rate of breast cancer in the United States grew from 84.8/100,000 to 112.4/100,000, an increase of 32.5%, with much of this increase being attributed to the greater use of screening mammography and earlier detection of breast cancer (2). Although there is hope that earlier detection will result in decreased mortality from breast cancer, so far, there has been no change in mortality rates according to Surveillance, Epidemiology and End Results (SEER) program data between 1973 and 1989 (3). In fact, breast cancer mortality has changed little since 1930 (1). Efforts to prevent the development of breast cancer through diet or other agents, as well as efforts to increase the earlier detection of breast cancer will be very important in the fight to decrease the incidence and mortality of breast cancer. However, many women will continue to be diagnosed with breast cancer and have spread of their disease beyond the breast either at the time of diagnosis or sometime later. Despite improvements in the adjuvant therapy of breast cancer, 20-60% of women with Stage II breast cancer will eventually relapse and die from their disease (4). Once breast cancer metastases become clinically detectable, the disease will ultimately claim the life of the patient in spite of the fact that it often responds to chemotherapy treatments. Thus, additional modalities of treatment for breast cancer are clearly needed. In addition to the standard modalities of surgery, radiation, chemotherapy and hormonal therapy, modulation of the immune system is another possible way of attacking breast cancer. This unique therapeutic modality offers the possibility of destroying breast cancer cells that are resistant to chemotherapy, hormonal therapy and radiation. During the last decade, major advances in our understanding of the immunobiology of cancer have established the feasibility of manipulating the host's immune response to tumor antigens in order to eradicate residual or metastatic cancer. This proposal is an effort to capitalize on these advances to develop an innovative new treatment strategy for breast cancer.

2. Involvement of T cells in the tumor response.

The importance of T cells in the anti-tumor immunity has long been appreciated (5). In animal models, T cells have been shown to be critical for the rejection of tumors induced by viruses, chemical mutagens, or ultraviolet irradiation (6-8). Consistent with this, athymic nude mice which are essentially devoid of functioning T cells are unable to reject even allogeneic tumors (9). Regarding human tumors, due to the limitations of experimentation, such direct evidence is harder to obtain. Nonetheless, T cell immunosuppression in humans is associated with an increased risk of cancer e.g. patients with AIDS are at risk for the development of Kaposi's sarcoma and non-Hodgkin's lymphoma and patients receiving immunosuppressive drugs after organ transplantation have an increased incidence of lymphoma and skin cancer (9).

Since T cells are an antigen-specific population of cells, their ability to induce the rejection of tumors implies that the malignant cells express specific antigens not found on other cells, and that in many instances these proteins are neo-antigens, as T cells would not necessarily generate a response against previously displayed self-proteins to which they had been tolerized. Although it was commonly held in the past that human cancers did not have tumor specific antigens, recent work has clearly demonstrated their existence. Indeed, a considerable body of evidence indicates that many tumors, including human breast cancers, express such tumor specific antigens (5, 9-14). For example, patients with breast cancer have been demonstrated to make antigen specific cytotoxic T lymphocytes (CTLs) that recognize certain epitopes of cell-surface mucin proteins that are phenotypically altered through aberrant glycosylation in more than 90% of breast cancers (15-17). The existence of tumor infiltrating lymphocytes which can lyse tumor cells provide another example of the ability of T cells to recognize tumor-associated antigens. Finally, an extreme instance of tumor-specific antigens occurs in the case of allogeneic tumors, which are almost uniformly rejected by immunocompetent hosts. This vigorous immune response may be due to the fact that alloantigens are intrinsically extremely immunogenic (i.e. a 10-100 fold larger percentage of T cells are capable of responding to alloantigens than to nominal antigens) (18).

If human cancers express unique antigens why aren't they rejected by the immune system? Recent advances in our understanding of the process of T cell activation may provide an answer to this question. It is now appreciated that complete activation of T cells requires 2 signals (19,20 and see section 3 below). The first is provided by antigen itself. The second or "co-stimulatory" signal is not

antigen specific but is normally provided by bone-marrow-derived antigen presenting cells (APCs) and is required for T cell responses. Moreover, while costimulation of T cells results in their complete activation, presentation of antigen in the absence of this costimulatory signal creates an anergic state whereby the T cells fail to respond normally to the antigen (21,22). Solid epithelial tumor cells (including breast cancer cells) lack the ability to deliver the costimulatory signal, and we postulate that this induces a state of unresponsiveness in the host that permits the tumor to grow unimpeded by the immune system. Indeed, it can be envisioned that this feature of the immune system, perhaps normally used to inhibit the development of autoimmune disease by preventing a response to self-antigens displayed on non-APCs, might be exploited by malignant cells of epithelial origin. Now that this requirement for a costimulatory signal is known, strategies to provide it can be developed in an attempt to create a vigorous rejection of the tumor by the immune system. The approach presented in this proposal is to use genetic transfection techniques to convert the tumor cell from delivering a tolerogenic signal to an activating signal.

3. The B7:CD28 interaction can provide a second signal.

As noted above, activation of T cells through the T cell antigen receptor (TCR) provides the first signal for T cell activation. This is sufficient to lead to entry of resting G0 cells into G1 phase of the cell cycle and result in expression of the cell surface high affinity IL-2 receptor (IL-2R). However, in the absence of additional signals, the IL-2 gene is not transcribed, T cells fail to proliferate, and appear to become anergic (reviewed in (19)). Thus, provision of antigen to T cells in the absence of co-stimulation is not a "neutral" event, but can lead to antigen-specific non-responsiveness.

APCs such as macrophages, dendritic cells and activated B cells are capable of providing second signals to T cells, synergizing with TCR stimulation and leading to complete T cell activation. The bulk of accumulated evidence indicates that a membrane bound molecule present on APCs is capable of providing costimulation to resting T cells or to Th1-type T cell clones (23, 24), and that a second signal is transduced when a T cell accessory molecule binds to its ligand on an APC. Recently, we and others have shown that the interaction between CD28 on T cells, and its ligand B7 on APCs is capable of delivering a co-mitogenic second signal (25-27).

a. CD28

CD28 is a 44 kd homodimeric glycoprotein member of the immunoglobulin gene superfamily. It is expressed on the surface of 80% of all peripheral T cells (95% of $CD4^+$ cells and 50% of $CD8^+$ cells) (28), and is the surface signal transducing element of a unique T cell activation pathway. Stimulation of T cells via the surface molecule CD28 can provide a second signal capable of synergizing with TCR stimulation to induce mitogenesis (28). Stimulation of the CD28 molecule strongly induces IL-2 production in TCR-stimulated T cells (28).

In addition to this co-mitogenic effect, CD28 stimulation enhances lymphokine gene expression 5-50 fold, even in T cells already maximally stimulated (29). This effect is mediated by specific stabilization of lymphokine mRNA (30). Signal transduction via CD28 also synergizes with the phorbol ester PMA to activate T cells independent of a rise in intracellular calcium (28). Perhaps due to its lack of dependence on calcium flux, T cell activation via CD28 is relatively resistant to the immunosuppressive effects of cyclosporine (28). Recently, we have shown that stimulation of the CD28 pathway in resting T cells and T cell blasts activates a protein tyrosine kinase which phosphorylates a distinct pattern of substrates than those which are induced following TCR/CD3 stimulation (31).

Finally, as noted above, previous studies have demonstrated that TCR engagement of T cells in the absence of accessory cells, or with accessory cells incapable of providing a second signal, can induce anergy (19). Upon identification of the CD28 pathway, it was postulated that co-stimulation via CD28 might block the development of anergy in this type of system. Harding et al. (32) have recently demonstrated this phenomenon showing that costimulation with anti-CD28 mAb prevents the induction of anergy in murine T cell clones stimulated with antigen in the absence of a second "costimulatory" signal.

b. B7

Like CD28, the B7 molecule is a member of the immunoglobulin gene superfamily. It was first defined as a B cell antigen expressed on activated and neoplastic B cells (33-35). However, B7 expression is not restricted to B-lineage cells, as B7 is inducible on IFNγ or LPS-treated monocytes (36). Thus the pattern of B7 expression parallels the ability of APCs to provide accessory function. Subsequently, B7 was shown to be a natural ligand for CD28 (25). Cloning of the B7 cDNA (35) and its expression in transfected CHO cells (25-26) has enabled studies of the results of CD28 stimulation by its natural ligand. These have shown that B7 binding to CD28 is capable of replicating many of the

previously observed actions of anti-CD28 mAb such as co-mitogenesis with anti-CD3 mAbs or PHA, stimulation of IL-2 gene expression, and induction of protein tyrosine phosphorylation (25-26, 31). Further study has also indicated that the cognate interaction between CD28 on $CD8^+$ cytotoxic T lymphocytes (CTLs) and B7 on target cells is an important component of the in vitro cytolytic response, and blockade of this interaction can significantly inhibit target cell lysis (37). These observations are important in light of recent data regarding the use of B7 to induce tumor specific immunity (see below).

c. CTLA-4

CD28 is encoded on human chromosome 2 (38). Immediately adjacent to the CD28 gene is a closely related gene, CTLA-4, which shares 32% amino acid identity with CD28 and has a similar genomic organization (39,40). Linsley and co-workers have produced a recombinant fusion protein, CTLA4Ig, which contains the extracellular domain of human CTLA-4 fused to a human immunoglobulin Cγ chain (27). In vitro, CTLA4Ig binds B7 with a Kd of 12 nM, approximately 20-fold greater that the avidity of the interaction between B7 and a CD28Ig fusion protein (200 nM). In addition, the only molecule that can be immunoprecipitated from ^{125}I-labeled cell lines with CTLA4Ig is B7. By flow cytometric analysis, CTLA4Ig, but not an isotype matched control fusion protein, binds to B7-transfected CHO cells, and neither protein binds to mock-transfected CHO cells.

4. Blockade of B7 strongly inhibits in vitro and in vivo immune responses: allograft rejection as a model.

It is readily appreciated that experiments that show that stimulation of CD28 with a natural ligand can induce in vitro immune responses are not the same as experiments that show an actual requirement for this interaction in vivo. Although CTLA4Ig was constructed with the extracellular domain of human CTLA4, it also binds to murine and rat B7 (personal communication, P. Linsley). This has allowed us to use CTLA4Ig to examine the role of B7 in rodent models of immune responses. To study the role of B7, we initially used rats and examined the events occurring during allograft rejection. First, we studied the in vitro response to alloantigen by testing the effects of CTLA4Ig on a one-way mixed lymphocyte culture (MLC) between Lewis rats ($RT1^l$, responder) and Brown-Norway rats ($RT1^n$, stimulator) (41). CTLA4Ig was able to block proliferation in a dose dependent fashion with virtually complete inhibition observed at a concentration of 1 μg/ml. This suggests that in order to mount a proliferative response in vitro, T cells must be stimulated not only through MHC engagement of the TCR but also require costimulation by B7 engagement of the CD28 receptor, and that CTLA4Ig can block this costimulatory event.

CTLA4Ig was next used in a rat model of organ transplantation to ascertain its ability to block immune responses in vivo (41). Recipient Lewis rats received a Brown-Norway heterotopic cardiac allograft and grafts were monitored by palpation. Untreated Lewis rats rejected the heterotopic Brown-Norway allografts in 6.8 ± 0.3 days. Next, animals were treated with daily injections of CTLA4Ig or an isotype-matched control monoclonal antibody L6 for 7 days. The allografts in all CTLA4Ig-treated animals remained functional following completion of drug administration (median survival time 30 days), whereas animals treated with the L6 control antibody uniformly rejected their grafts by day 8 ($p<0.0001$).

5. Other ligands for CD28.

Recently, several groups have reported that B7 is not the sole ligand for CD28/CTLA4, but rather that there is at least 1 related gene, termed B7-2 or B70, which can also function as a stimulatory ligand for CD28 (42-45). The expression pattern of B7-2/B70 differs somewhat from B7 in that it is expressed at higher levels on antigen presenting cells, and is induced at earlier time points in activated cells. From a functional standpoint, there is no known difference between activation of the CD28 pathway via ligation with B7 versus ligation with B7-2/B70. Both CD28 ligands provide co-stimulatory signals to T cells. While the distinct physiologic roles of B7 and B7-2/B70 remain to be elucidated, it is clear that both molecules can provide second signals to T cells. While the choice to use B7 for adoptive immunotherapy was based on its identification at the time as the only ligand for CD28, the data regarding the use of B7 to induce tumor rejection (Townsend, Chen, Baskar, and our own preliminary data - see below) validates this choice.

6. Therapeutic approaches to induce tumor immunity.

a. Systemic IL-2 +/- LAK cells

The immune response to foreign antigens involves a coordinated release of cytokines from T cells which induces the clonal expansion and/or activation of a variety of effector cells including CTLs,

B cells, NK cells, and macrophages. A variety of strategies have been devised to induce tumor-specific immunity based on the notion that defective production of cytokines may play a role in the failure of animals to "reject" their tumor. Thus, exogenous systemic administration of lymphokines such as IL-2 with or without adoptively transferred lymphokine activated killer cells (LAK cells) can be an effective form of anti-tumor therapy for patients with renal cell carcinoma, melanoma or lymphoma (46). Although the exact mechanism of action of IL-2 is not known, it is well established that IL-2 has no direct antiproliferative activity, so its ability to induce tumor regressions in patients with advanced disease indicates it is working indirectly, perhaps by enhancing the immunogenicity of the cancer, the cytotoxicity of immune effector cells or through the induction of other directly antiproliferative cytokines. Unfortunately, systemic administration of high doses of IL-2 is associated with severe side effects and many prevelant cancers such as colon, lung and breast cancer fail to respond significantly to this treatment.

b. Systemic IL-2 + Tumor Infiltrating Lymphocytes (TILs).

Animal experiments demonstrated that TILs ,which are antigen specific cytotoxic lymphocytes, are 100 times more potent than the non-MHC restricted LAK cells in adoptive immunotherapy studies (47). Culturing TILs with IL-2 resulted in their expansion and enhancement of their ability to kill in an antigen restricted fashion (47). In mice, these in vitro stimulated TILs mediated tumor regression when adoptively transferred into syngeneic hosts inoculated with the specific tumor cell line from which the TILs were originally derived (47). Preliminary results using a similar approach in patients with metastatic melanoma have yielded promising results (48). Responses appear to correlate with the number of TILs administered and to the specific cytotoxicity in vitro of the TILs to the autologous tumor (49,50). However, therapy utilizing the adoptive transfer of TILs is technically cumbersome, expensive, and fails to produce TILs in approximately one third of the patients. The vaccination approach proposed in this grant application could also lead to the development of TILs in the draining lymph nodes (these will be assayed for - see Immunological Monitoring under the Methods section). Future therapeutic protocols could attempt to harvest and expand those TILs for adoptive immunotherapy.

c. Vaccination with genetically altered cells

Gene transfer into tumor tissue has become a potentially attractive means of enhancing the immune response to the tumor. To date, a variety of cytokine genes including IL-2, TNF, IFNγ, GM-CSF, and IL-4 (51-57) have been transfected into tumor cells ex vivo in an attempt to overcome the lack of effective T cell stimulation by creating a local continuous secretion of a cytokine that will activate T cells, recruit other inflammatory cells into the area and possibly induce other cytokines to be secreted. Animal studies have shown that this approach enhances the immunogenicity of the tumor, limiting its outgrowth and in some cases, causing established tumors to regress. Importantly, in some instances (IL-2, TNF, and IL-4), mixing both non-transfected and transfected cells prior to injection at a single site resulted in regression of both cell populations, indicating that the induced immunity was locally active. Another approach is to directly transfect a gene in to a tumor site that encodes a foreign MHC molecule to enhance the recognition of the tumor cell with the hope of increased local cytokine production and T cell activation (58). The early toxicity trials of these gene therapy approaches are currently underway.

The strategy we will pursue in this proposal will be to genetically alter breast cancer cells to express a gene which encodes for a cell surface protein, B7 which provides a potent costimulatory signal for $CD4^+$ cells to produce a variety of lymphokines, and which also appears to be capable of directly stimulating $CD8^+$ CTLs to lyse targets. The advantage this approach might have over the expression of a single cytokine gene is it should recreate the normal scenario by which the immune system detects and destroys cells bearing foreign antigens. That process involves a complicated orchestration of the production of multiple cytokines and cell-to-cell interactions. We feel that transfecting a gene to provide a costimulatory signal to T cells will more likely result in the coordinated secretion of multiple cytokines in the proportions that are efficacious in generating immunity and therefore, may be more therapeutically effective than transfecting a single cytokine gene. This precise strategy (transfection of B7) already has been used in animal experiments by other groups to induce tumor specific immunity to melanoma or to sarcoma (59-61). Our own preliminary studies indicate that this approach is also successful in inducing rejection of murine breast cancer cell lines (see below).

7. Use of B7 to induce tumor rejection.

Three different groups of investigators have conducted animal experiments that demonstrate an important role for B7 in inducing anti-tumor immunity in vivo. Townsend and Allison (59) transfected a K1735 murine melanoma cell line with murine B7 and injected it into syngeneic mice. Compared to a control vector-transfected K1735, the B7-transfected tumor grew less well as mice injected with the B7-expressing tumor were able to "reject" the malignant cells and appeared disease free. Furthermore, prior exposure to the B7-transfected tumor cells was able to protect the host mice from a subsequent challenge of the parental tumor cell line. The effect appeared to be dependent on the presence of $CD8^+$ T lymphocytes, because B7-expressing cells grew readily in mice depleted of this subset, with a tumor growth rate the same as that observed with the parental cell line or the vector-transfected control tumor cell line. Depletion of $CD4^+$ T cells did not affect the ability of the host animals to reject their tumor.

Chen et al. (60) used the K1735 cell line that was further modified by insertion of a xenogeneic viral antigen, the E7 gene product of human papilloma virus. They transfected this modified cell line with B7 and showed that immunization with the B7-transfected cell line protected animals from subsequent challenge of the parental tumor cell line and also from challenge with nonB7-transfected tumor cells, indicating that it may not be necessary to transfect all of the tumor cells with B7 to elicit an immune response against the established tumor. Again, the antitumor activity seemed to be mediated by $CD8^+$ T lymphocytes. These investigators also showed that injection of B7-transfected tumor cells in one flank resulted in the complete rejection of nonB7-transfected tumor cells placed simultaneously in the other flank. Finally, this treatment was shown to eliminate established pulmonary micrometastases from non B7-transfected tumor cells.

In the third study (61), Glimcher and colleagues demonstrated that mouse sarcoma cells genetically engineered to express B7 stimulated potent tumor-specific T cells that caused rejection of both transfected and native neoplastic cells. A notable difference between this study and the other two was that $CD4^+$ T cells were responsible for the anti-tumor activity in this model. It is also noteworthy that this study utilized sarcoma cells rather than melanoma cells, thus showing that the use of B7 to induce tumor rejection can be extended to tumors other than melanoma.

In summary, these studies make several important points. First, B7 expression by itself can lead to tumor rejection. Second, rejection of a B7-positive tumor can lead to protective immunity against a B7-negative tumor. Third and most important for clinical use, exposure to a B7-expressing tumor can lead to rejection of previously established metastatic B7-negative tumor.

8. Use of B7 to induce protective immunity against breast cancer.

Our collaborators James Wilson and Steven Eck at the University of Pennsylvania have performed a series of preliminary studies designed to examine the use of ectopic B7 expression in a murine model of breast cancer. SCK cells (J.G. Rhee, University of Maryland, Baltimore, MD) arising from a spontaneously mammary carcinoma in A/J mice ($H\text{-}2^a$) is a murine mammary carcinoma cell line that grows in culture as well as forms lethal tumors in syngeneic hosts. After subcutaneous inoculation of as few as 1.0×10^3 viable SCK cells into 6-8 week-old, female, syngeneic mice tumors are consistently detectable within three weeks. Death or the need for euthanasia inevitably follows about 7 to 10 days later.

SCK cells normally do not express cell surface B7 antigen. To test the effect of ectopic B7 expression on in vivo tumor cell growth, SCK cells that constitutively express murine B7 (mB7) were generated. A cDNA encoding normal mB7 (from Louis Lanier, DNAX, Palo Alto, CA) was inserted into the pM 6 proviral vector. pM 6 and pMV6mB7 were transfected into ψCre packaging cells to generate ecotropic retroviruses, which were used to infect SCK cells. Transduced SCK cells were selected in media containing 400 ug/ml G418 (Gibco), and >200 colonies of surviving cells were pooled to maximize the likelihood of deriving lines that reflect the heterogeneity of the parental line. Flow cytometric analysis using a rat monoclonal antibody (mAb) to mB7 (Pharmagen, San Diego, CA) demonstrated that the MV6mB7-transduced SCK cells but not the MV6-transduced SCK cells expressed surface mB7.

mB7-SCK-M 6 cells and mB7+ SCK-M 6mB7 cells are indistinguishable morphologically and have similar in vitro growth characteristics, but differ significantly in their ability to form lethal tumors in syngeneic A/J mice. Injection of 5×10^3 viable mB7-was uniformally tumorigenic and lethal (6/6); whereas 5×10^3 mB7+ SCK cells resulted in no mice developing lethal tumors (0/6). Since mice injected with 0.5×10^3 SCK cells consistently developed tumors, the survival of most mice receiving 5×10^3 mB7+ SCK cells is unlikely to be due to inadvertent injection of too few cells. These

findings suggest that mB7 expression by SCK cells impairs their ability to form tumors in syngeneic hosts.

These studies indicate that the utility of B7 can be extended to breast cancer as well, and provide the impetus to move to clinical trials.

9. Use of primary human breast cancer cells for gene therapy.

The feasibility of gene therapy for breast cancer depends critically on the ability to isolate human breast cancer cells from primary tumors and/or metastatic sites in a manner suitable for gene transfer to be carried out with these cells. With the approach that we have chosen, in which a key immunoregulatory molecule is expressed in a cell in order to induce an immune response to that cell, it is particularly important that we be able to eliminate contaminating normal cells from tumor specimens in order to minimize the risk of "vaccinating" patients with B7-expressing normal cells, as this might potentially lead to a deleterious autoimmune process. One of the most difficult tasks in attempting to culture human breast cancer cells from primary tumors is related to the rapid proliferation of normal mammary epithelial cells that emerge in these cultures (62,63). Primary human breast cancers are a mix of normal and neoplastic mammary epithelial cells, and normal cells present in these samples grow very rapidly in culture in a way that precludes isolation of the neoplastic cells (64). In addition, using growth factor combinations that stimulate proliferation of normal mammary epithelial cells does not stimulate rapid growth of breast cancer cells. One means to circumvent this problem is to obtain breast cancer cells from metastatic sites where they will be free of contamination by normal mammary cells. We plan to utilize skin and nodal metastases, as well as malignant ascites and pleural effusions as sources from which to isolate breast cancer cells.

As a prelude to this proposal we have also assessed the expression of MHC class I and class II antigens on human breast cancer cells, since a strategy designed to provide a co-stimulatory signal will only succeed if breast cancer cells also express MHC. Thus, it was important to verify that breast cancer cells which might produce neo-antigens, express MHC molecules which would be capable of presenting these antigens to T cells. Our studies using 3 freshly obtained human breast cancers demonstrate that all 3 express abundant amounts of MHC class I molecules on their cell surface (as assessed by flow cytometry using the anti-HLA class I framework Ab W6/32 - data not shown). Two of the 3 cancers also expressed MHC class II molecules (detected by the anti-HLA DR framework Ab L243).

10. Isolation of Human Breast Cancer Cells.

Novel methods of breast cancer gene therapy that make use of expression vectors encoding genes important for immune functions will require the development of methods for the isolation and transfection of primary human breast cancer cells. It is well known that primary human breast cancer cells are among the most difficult human cancer cells to culture in vitro. As a result of this difficulty, only a relatively small number of human breast cancer cells lines have been developed and the majority of those lines have been derived from pleural effusion specimens of patients with advanced breast cancer (65,66). Over the past several years, methods for in vitro growth of human breast cancer cells have improved substantially. These improvements have resulted in the development of new breast cancer cell lines from our laboratory as well as from other labs (67-72). However, the majority of patient-derived human breast cancers still do not yield long-term cell lines. Our own experiments in this area indicate that there are a number of reasons for the relative inability to culture primary human breast cancer cells. First, the hormone and growth factor requirements for growth of primary human breast cancer cells in vitro are still not well characterized. The fact that breast cancer cells from different patients express different requirements for exogenous hormones and growth factors adds a layer of complexity to these studies. A second aspect of breast cancer cell growth that presents an obstacle for their growth in vitro concerns the rate at which these cells proliferate in culture. Human breast cancer cells growing in vivo exhibit volume doubling times ranging from 100 to greater than 500 days and have cell cycle times of greater than 100 hours (73-77). These long cell cycle times are maintained when breast cancer cells are transferred to in vitro culture (68, 64). In contrast to cancer cells, normal cells when put in culture and exposed to hormones and growth factors required for cell proliferation, grow with doubling times of 24 to 36 hours. Since most culture conditions that have been employed to grow breast cancer cells were originally developed to support growth of normal fibroblasts or epithelial cells, the rapid proliferation of normal cells that takes place within these cultures severely compromises the ability to culture breast cancer cells under these conditions. In recent studies we demonstrated that culture media that are expressly designed to support growth of normal luminal mammary epithelial cells (the cell type from which breast cancer arises) yields rapid

overgrowth of normal luminal mammary cells even in cultures initiated from primary human breast cancer specimens (68). Thus, it is clear that successful culture of human breast cancer cells will not only require optimal culture conditions, but will also require cell isolation methods that allow breast cancer cells that grow with long doubling times to not be overgrown by normal cells. In fact, the development of breast cancer cell isolation methods may be of greater importance to gene therapy studies than the development of methods for the growth of the cells in vitro.

Another difficulty eluded to above is that even successful cultures of human breast cancer cells grow with very long population doubling times especially at low passage. Thus, the use of expression vectors that yield stable integration and expression of a transgene will be difficult in such situations as transfection efficiencies are dramatically influenced by the growth rate of the cells. An alternative strategy for expression of a transgene in human breast cancer cells involves the use of adenoviral expression vectors that yield high efficiency transient expression of a transgene. Unlike plasmid vectors or retroviral vectors, adenoviral vectors express the transgene without stable integration of the gene into the genome. Thus the low replication rate of human breast cancer cells in vitro will not hinder gene expression in cells infected with these vectors. However, the use of these non-selective vectors in the context of a gene therapy protocol will require that breast cancer cells be isolated and separated from other cell types prior to infection so as to minimize as much as possible the likelihood of unintentionally transducing "normal" cells with the expression vector. One potential method for the selective isolation of human breast cancer cells from mixed cell populations involves the use of antibodies that differentially recognize human breast cancer cells from normal mammary epithelial cells and other normal cell types, and then employing magnetic beads conjugated to secondary antibodies. Although there has yet to be described a true human breast cancer specific antigen, there are now several reports that indicate that human breast epithelial cells express mucins that consist of repeating units of 20 amino acids that are normally heavily O-glycosylated (78). Human breast cancer cells fail to properly gycosylate theses breast epithelial mucins resulting in exposure of cryptic antigenic determinants not exposed on normal mammary epithelial cells (79). Monoclonal antibodies have been developed against the core peptides of these epithelial mucins and these antibodies differentially recognize breast cancer cells from normal mammary epithelial cells (80-82). One such antibody, Sm-3, reacts with over 90% of breast cancer cells while showing no reactivity to normal mammary epithelial cells (80).

The availability of antibodies that differentially recognize human breast cancer cells raises the possibility of using these antibodies to purify the cell populations that express the epitope. Magnetic beads conjugated with anti-mouse or anti-rabbit antibodies are now commercially available and can be used to isolate cell populations that have been exposed to antibodies. These magnetic bead separations result in the generation of highly pure populations of cells that remain viable. In our laboratory, we have used beads coated with the mouse monoclonal antibody Tab 254 (raised against the extracellular domain of the erbB-2 protein) to isolate erbB-2 overexpressing human breast cancer cells. We have also used beads coated with the Mc-5 antibody which recognizes a breast epithelial mucin, to isolate breast cancer cells from mixed populations obtained from metastatic breast cancer specimens. Similar cell separation methods are also being developed that make use of biotinylated primary antibodies and an avidin-affinity matrix in place of the magnetic beads. Cells that have been exposed to biotinylated primary antibody are then bound to the avidin-matrix and non-bound cells are washed away. The bound cells can then be eluted from the matrix. The advantage of this method is that cells isolated in this way do not have magnetic beads bound to them which may interfere with certain applications.

If one can selectively isolate human breast cancer cells in a viable state, it should be possible to infect those cells with adenoviral expression vectors. These vectors infect epithelial cells with high efficiency and express the transgene in a transient fashion. This transient expression eliminates the need for rapidly replicating cells required for stable integration and expression following infection with retroviral expression vectors. Furthermore, since initiation of immune responses takes only a few days and expression of B7 is required only during the first 24-48 hours, it appears likely that the fact that the tumor cells will express B7 only transiently will not compromise the ability to assess the efficacy of this form of immunotherapy.

In preliminary experiments utilizing an adenoviral expression vector containing the LacZ reporter gene, we found that human breast cancer cell isolates were readily infected with this vector. At multiplicities of infection of 100 pfu per cell, greater than 80% of breast cancer cells expressed the transgene 24 hours after infection and this expression level was maintained for greater than one week in vitro.

In the experiments proposed in this application, we will continue to develop and optimize methods for the selective isolation of human breast cancer cells using a panel of monoclonal antibodies. Experiments will also be carried out to refine the methods for infection of purified human breast cancer cells using adenoviral expression vectors and to characterize the expression of transgenes in the infected cells.

II. Hypothesis/Purpose

The working hypothesis for the proposed studies is that immune responses against breast cancer cells are incomplete **not** because these cells fail to express tumor specific antigens or MHC molecules, but rather, because the cells do not express molecules such as B7 which are required in conjunction with MHC to induce a full immune response. Thus, induction of B7 expression in breast cancer cells by transduction with appropriate expression vectors and re-innoculation of B7 expressing tumor cells may result in induction of a complete immune response against the transfected cells as well as the non-transfected counterparts derived from the same cell population.

III. Technical Objectives.

Our collaborators, Drs. James Wilson and Steven Eck at the University of Pennsylvania, are developing adenoviral expression vectors for the human B7 gene. Using these expression vectors, the specific aims of our proposal are to:

1. Develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer.

2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells used as a vaccine to enhance the immune response to the tumor.

4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

IV Methods.

Specific Aim #1. Selective isolation of human breast cancer cells from primary tumors and metastases using monoclonal antibody-conjugated magnetic beads.

Rationale. In order to isolate a highly pure population of human breast cancer cells that are suitable for infection with adenoviral expression vectors it is necessary to prepare a viable single cell suspension of cells from breast cancer specimens. In our previous studies with primary and metastatic human breast cancer specimens we have used an enzymatic dissociation procedure to prepare breast cancer cells for cell culture experiments (68,69,83). For cell culture applications, generation of cell suspensions that consist of multi-cell aggregates of breast cancer cells and normal cells is sufficient and even advantageous in some ways. For the experiments to be performed in the present studies, the cell suspensions obtained following enzymatic dissociation of breast tissue specimens will be treated further to prepare single cell suspensions. Single cell suspensions prepared from these specimens will be exposed to a panel of monoclonal antibodies in order to separate normal cells from malignant cells. Finally, the isolated breast cancer cells will be infected with adenoviral expression vectors, containing either a reporter gene (LacZ) for developmental studies, or the B7 gene for gene therapy experiments.

a. Preparation of single cell suspensions of human breast cancer cells.

Solid tumor specimens, either primary tumors or solid metastatic nodules, will be minced with sterile scalpels until tissue pieces are approximately 1 mm^3. The minced tissues will be suspended in Medium 199 containing Worthington type III collagenase (Worthington Chemical Co., Freehold, NJ) at a concentration of 200 units per ml, and Dispase (Boehringer-Mannheim, Indianapolis, IN) at a concentration of 1 mg per ml. Twenty mls of enzyme solution are used per gram of tissue. The tissues are incubated overnight in a 37° water bath shaking at 65 rpm. The next day, the remaining tissue clumps are mechanically dissociated by repeated pipetting of the suspension. The cells are then

washed three times by centrifugation at 250 x g and re-suspended in fresh Medium 199 after each wash. This enzymatic dissociation procedure results in a mixed suspension of single cells, small aggregates and large mammary organoids. The viability of the cells in this suspension is greater than 95%. To prepare a single cell suspension from the mixed aggregate population, the cells will be washed in Ca^{++}, Mg^{++}-free, Hanks balanced salt solution (CMF-Hanks BSS) and then incubated for 4 hours in CMF-Hanks BSS containing 10 mM EDTA, at 4° with gentle rocking. The cells will be mechanically dissociated every hour during the four hour period by repeated pipetting of the cell suspension. If necessary to maintain viability of the cells during this incubation, the CMF-Hanks-EDTA solution will be supplemented with 5% fetal bovine serum that had been treated with Chelex to remove divalent cations. After the four hour incubation, single cells are separated from any remaining cellular aggregates by filtration through Nitex mesh with a 20 um pore size.

Our preliminary data indicate that collagenase/Dispase dissociation of breast tissues does not adversely effect the integrity of cell surface molecules as these aggregates are quite reactive to antibodies directed against cell surface proteins. In generation of single cell suspensions, however, it is imperative that a method be used that does not alter the peptide epitope present on the surface of breast cancer cells. For this reason, we have chosen to use chelating agents that disrupt cell to cell interactions without degrading cell surface molecules to achieve the final single cell suspensions. It is necessary to obtain single cell suspension for the final cell purification procedures as the separation methods make use of antibodies that bind to epitopes expressed on beast cancer cells and not on normal mammary epithelial cells. If cell aggregates are used in the cell isolation procedures and if aggregates contain both normal and neoplastic cells, then the purpose of using breast cancer specific antibodies would be defeated.

b. Isolation of breast cancer cells using antibody conjugated magnetic beads.

The basic strategy for isolating breast cancer cells involves the use of magnetic beads (Dynabeads, Dynal Inc. Great Neck, NY) that have been conjugated with anti-mouse IgG antibodies. Thus, the anti-mouse antibodies on the beads can be bound to mouse monoclonal antibodies directed against cell surface epitopes to prepare a reagent that specifically binds cells expressing the epitope. Following incubation of a cell suspension with antibody coated magnetic beads, the bound cells can be separated from non-bound cells by placing the tube in magnetic particle concentrators (MPC) designed to hold microfuge tubes. The beads and bound cells adhere tightly to the wall of the MPC and the non-bound cells are aspirated from the tube. The tube is then removed from the MPC, the cells re-suspended in medium and this washing procedure is repeated three to four times. With this method, we have separated mixed cell populations with greater than 99% efficiency using antibodies against the erbB-2 protein and antibodies against breast epithelial mucins. The cells isolated in this way have been seeded into culture and exhibit high viability as indicated by their ability to attach in culture and proliferate.

To coat magnetic beads with mouse monoclonal antibodies, 1×10^8 anti-mouse IgG Dynabeads are suspended in 1 ml of CMF-Hanks BSS and incubated with 1 ug of mouse monoclonal antibody with rocking at room temperature for two hours. Following the incubation, the beads are washed extensively with CMF-Hanks BSS by adhering the beads to the tube wall using the MPC, aspirating the medium, re-suspending the beads in 1 ml of fresh medium and incubating with rocking for 30 minutes. This washing procedure is repeated three times. After the last wash, the beads are suspended in CMF-Hanks BSS at a concentration of 10^8 beads per ml.

To isolate cells using antibody conjugated magnetic beads, 2×10^7 magnetic beads are added to a 1 ml aliquot of a cell suspension of 1×10^7 cells and incubated with rocking at room temperature for two hours. Next, the cells bound to the beads are washed three times to separate them from non-bound cells. If necessary, the beads can be removed from the purified cells either by trypsinization or by incubation with the peptide epitope that was used to generate the primary antibody. The cells isolated in this way can be used to initiate cell cultures of breast cancer cells or can be infected with adenoviral expression vectors. For the experiments to be carried out in this project, we will employ magnetic beads coated with three different antibodies. The first antibody, Sm-3, was generated against the core peptide of breast epithelial mucins (80). As discussed earlier, altered glycosylation of mucins that occurs in greater than 90% of breast cancer cells reveals the peptide epitope that is masked in normal cells by glycosylation. Thus, the Sm-3 antibody coated beads will be the primary antibody for isolating human breast cancer cells from primary tumor specimens that contain both normal and neoplastic mammary epithelial cells. The Sm-3 antibody was obtained from Dr. Joy Burchell, Imperial Cancer Research Fund, London, UK. A second antibody, Mc-5, recognizes breast epithelial mucins expressed on virtually all breast cancer cells. This antibody also binds to normal mammary

epithelial cells making it less useful for primary tumor specimens. However, magnetic beads coated with this antibody have been used in our laboratory to isolate breast cancer cells from metastatic lymph nodes and pleural effusion metastasis. This antibody was obtained from Dr. J. Peterson, Cancer Research Fund of Contra Costa, Walnut Creek, CA. Finally, a third antibody, Tab-254, binds to the extra-cellular domain of the erbB-2 protein. Magnetic beads conjugated with this antibody are useful in isolating breast cancer cells from primary or metastatic sites that overexpress the erbB-2 protein as a result of amplification of the c-erbB-2 (Her-2/neu) gene, which occurs in approximately 30% of breast cancer cases. A panel of Tab antibodies against the erbB-2 protein have been obtained from Dr. Beatrice Langton, Berlex Biosciences, Richmond, CA.

Specific Aim #2. Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA.

Rationale. Prior to the use of transduced breast cancer cells as immunotherapy the methodologies to transduce the maximum number of cells with an adenoviral vector and to verify that large numbers of cells express the vector-encoded human gene for at least several days must be developed. In this specific aim, we will perform a series of experiments aimed at optimizing methods for the infection of purified human breast cancer cells with adenoviral expression vectors and for optimizing the expression of a transgene within these vectors. Experiments will also be performed to determine the immunogenicity of human breast cancer cells that express the B7 gene following infection with appropriate adenoviral expression vectors.

a. Infection of purified human breast cancer cells with adenoviral expression vectors.

To perform the optimization experiments, an adenoviral vector containing a reporter gene (LacZ) as the transgene will be used. In preliminary studies with early-passage breast cancer cell lines developed in our laboratory, we have found that overnight exposure of these cells to these adenoviral vectors results in expression of the LacZ transgene in greater than 80% of the cells. To optimize infection of purified human breast cancer cells with the adeno-LacZ virus, aliquots of 10^6 human breast cancer cells, purified using methods described above, will be incubated in suspension with adenoLacZ virus for 24 hours with gentle agitation. Multiplicity's of infection ranging from 10^2 to 10^4 pfu's per cell will be tested in these experiments. Following infection, cells will be seeded into culture using media that we have developed for human breast cancer cell growth, and the cells will be assessed for LacZ activity at 24 hours, 3, 7, 10 and 14 days after infection. This experiment will be carried out with cells from at least 10 separate breast cancer patients. In these experiments, we will determine the optimum multiplicity of infection and the duration of the transgene expression in purified human breast cancer cells infected with adenoviral vectors immediately after their isolation.

b. Infection of purified human breast cancer cells with B7-adenoviral vectors.

Experiments will then be performed to transduce human breast cancer cells with the human B7 gene using adenoviral vectors developed by James Wilson and Steve Eck (see letter of consultancy). For these experiments the conditions shown to yield optimal transduction of the LacZ reported gene will be used for B7. Expression of B7 protein on the surface of the human breast cancer cells will be assessed by flow cytometry using CTLA4Ig or commercially available mouse anti-human B7 mAb. We will also verify that the cells are capable of supporting B7-mediated responses such as providing co-stimulatory signals (as assessed by proliferation and IL-2 gene expression) for autologous T lymphocytes activated with phorbol ester, bacterial superantigens, or PHA (methods as outlined under specific aim #4). Each of these is an accessory cell dependent stimulus, however we and others have shown that purified T cells can respond to these stimuli in the presence of B7-transfected CHO cells. Thus we will use B7-transfected CHO cells as a positive control in these studies. This also will allow us to compare the relative co-stimulatory abilities of $B7^+$ CHO cells and autologous $B7^+$ breast cancer cells. Since in the design of our phase I study (specific aim #3) the cells will be irradiated with 5000 cGy prior to injection into patients, we will also verify in these studies that the cells retain costimulatory capacity after irradiation at this dosage.

Specific Aim #3. Conduct a Phase I clinical trial assessing the toxicity of autologous B7-transduced irradiated breast cancer cells as a vaccine to enhance the immune response to the tumor.

Rationale and Overview In order to test the safety of a genetically modified autologous tumor vaccine, a phase I clinical trial must be performed. Although other cancer patients have received genetically altered tumor cell vaccinations (transfected with cytokine genes), this trial would represent

one of the first times a genetically altered tumor cell vaccine that contained the gene for B7 was given. Therefore, it is prudent to begin the testing in patients with advanced refractory breast cancer. This is not the optimal setting for a vaccine to work, since these patients will have a significant tumor burden and have received several chemotherapy regimens, both of which can significantly depress the immune system. Ideally, a vaccine should be utilized to treat patients with low tumor burdens such as is the case after primary definitive surgery and/or irradiation. Nevertheless, the initial toxicity and safety studies must always be performed in patients with advanced disease and limited life expectancy. We plan to administer an autologous irradiated B7-transfected breast cancer cell vaccine once to patients with advanced breast cancer and observe them closely for side effects, toxicities, any clinical anti-tumor responses, and changes in their immune response to the tumor. Cohorts of six patients each will receive one of 3 dose levels (numbers of transfected breast cancer cells transfected) in an escalating fashion as toxicity permits. Assuming toxicity is not severe, future clinical trials could consider a vaccination schedule with several planned vaccinations, as well as the addition of adjuvants (if necessary) or systemic immune stimulating agents such as IL-2 following the vaccine. Future studies could also incorporate the harvest of regional lymph nodes and expansion of TILs for adoptive immunotherapy. The details of the proposed clinical trial are described in the model protocol that is in Appendix F. The key features will be delineated below. We recognize that certain technical details are still in the developmental phase. These details will be incorporated into the final version of the protocol prior to submission to the IRB, RAC and FDA. We also wish to emphasize that the appropriate animal toxicity studies will be performed prior to the initiation of the clinical trial by our collaborators Drs. Wilson and Eck (at the University of Pennsylvania) using the type of B7-expression vectors that would be used in our human studies. These toxicity experiments will include intravenous injection of the B7 vector, as well as deliberate transduction of a variety of non-malignant cells such as hepatocytes, fibroblasts and keratinocytes. The studies will assess the animals for adverse effects including the induction of unwanted autoimmune responses.

Phase I Study Objectives.

1. To determine the toxicity of subcutaneously administered irradiated autologous breast cancer cells that have been transfected with the human gene for B7 in patients with advanced or metastatic breast cancer.
2. To determine the maximum number of transfected breast cancer cells that can be safely given to these patients.
3. To determine if the vaccination results in an immune response and to characterize that immune response.
4. To observe patients for any antitumor responses.

Eligibility Requirements.

Patients must have advanced breast cancer that has failed to respond to at least two standard chemotherapy regimens used in the metastatic setting and who are considered unlikely to benefit from further salvage chemotherapy regimens or hormonal regimens. They must also have a source of autologous tumor that can be easily harvested. This includes patients with subcutaneous or cutaneous metastases, patients with easily excisable lymph nodes containing metastatic tumor, and patients with malignant pleural effusions or ascites. Patients must have a good performance status and a life expectancy of at least three months. Patients must be at least 18 years old. *There is no exclusion for sex or ethnic background.* Patients must have evaluable or measurable disease in addition to the disease that will be surgically removed for the purposes of formulating the autologous vaccine. Adequate baseline organ function will be required. In addition, patients must not be anergic to standard recall antigens. Patients may not have received prior antitumor vaccines or immunotherapy. Patients will be excluded if they have any autoimmune diseases, evidence of HIV infection or AIDS, active infection, bleeding, pregnancy, or lactation, or any significant uncontrolled medical or pyschiatric illness. Patients who require corticosteroids or anticoagulation are ineligible.

Study Design.

Patients will undergo surgical removal of metastatic disease under local anesthesia in order to provide autologous tumor cells that can be transfected with the human B7 gene. A section of the removed tumor will be sent to surgical pathology for pathologic diagnosis. The remainder of the specimen will go to the laboratory to prepare B7-transfected autologous tumor, for immunologic assays, and for cryopreservation. (For details on the purification and transfection of the breast cancer cells see the technical methods section for specific aim 1 and 2). After the autologous breast cancer cells have been transfected with B7 they will be irradiated with 5000 cGy, a dose of radiation that renders them nontumorigenic but allows them to remain metabolically active. They will then be

injected intradermally into the thigh approximately 10 cm below the inguinal lymph nodes and the injection site will be marked with an ink tatoo for future biopsy. The injections will be administered in the Clinical Research Center, University of Michigan Hospital and the patients will remain in the hospital over night. Cohorts of six patients each will be treated with escalating doses of autologous irradiated B7 transfected breast cancer cells according to the following scheme: a): 10^6 cells, b): 10^7 cells, c): 10^8 cells (for technical reasons, 10^8 cells is the likely maximum number of cells that could be obtained from these patients). Individual patients will receive one dose level of cells (i.e. there is no intrapatient dose escalation). Only one vaccination is planned for each patient unless the patient demonstrates clinical benefit from the treatment, whereupon the patient can receive additional monthly vaccinations as long as such benefit persists. Each patient will be observed for at least three weeks at a given level of cell injection before the patients are permitted to enroll on the next higher dose level of cells. If one or fewer patients experience dose-limiting toxicity at a given number of cells injected, escalation will be permitted to continue to the next level. If two or more patients sustain dose-limiting toxicity, then that level of cells will be determined as the dose-limiting number of cells and the dose level of cells below that will be defined as the maximum tolerable dose of cells to be injected. It is possible that at the maximum dose of B7-transfected cells dose-limiting toxicity will not be observed.

Once escalation is completed, a separate cohort of six patients will be treated with both B7-transfected autologous irradiated breast cancer cells and vector only-transfected autologous irradiated tumor cells. One injection will be placed in the left thigh and one in the right thigh at the same time. The purpose of treating this cohort of patients is to compare the immunologic response at the vaccination site and in the draining lymph nodes from one leg to the other. This will help determine if B7 transfection enhances the immune response above that which is seen with transfection of vector alone.

Study endpoints.

TOXICITY - Patients will be closely followed and observed for the development of any clinical side effects from the treatment. Toxicity will be graded according to the Cancer Treatment Evaluation Program toxicity scale. The major toxicity that is anticipated is local redness, swelling, pain, and increased warmth at the injection site. Patients will be monitored for the development of clinical symptoms suggesting autoimmune disease or allergic reactions. Changes in laboratory parameters (complete blood count, chemistry panel, coagulation studies, urinalysis, as well as tests for the development of autoimmune disease [ANA, RF, CH50, anti-DNA abs, T4, TSH]) will be assessed two and four weeks after vaccination and thereafter once/month. In addition, sera and peripheral blood mononuclea cells will be obtained for archival purposes according to the current safety monitoring guidelines by the Center for Biologics Evaluation and Research (presently, once/month on treatment and every three months thereafter).

IMMUNE RESPONSE - A biopsy of the vaccination site along with surgical removal of one to three draining inguinal lymph nodes will be performed two weeks after the vaccination. Peripheral blood will be obtained at two weeks and 4 weeks and then once/month. DTH skin testing will be performed monthly. The details of the immunologic monitoring are extensively described in the methods section for specific aim #4.

ANTITUMOR RESPONSE - Four weeks after vaccination, the patients will undergo reevaluation to determine if their disease has responded or progressed using standard response criteria. Patients whose disease has not worsened or has regressed (even if it does not meet the criteria for partial regression) will be eligible to receive additional cycles of treatment using the autologous irradiated B7-transfected cancer cells providing that they experienced no severe toxicity with the first vaccination. Patients may continue this treatment until they have evidence of progressive disease.

Specific Aim #4. Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response.

Rationale.

It is our hope that the phase I clinical trial (specific aim #3) will demonstrate that administration of B7-transfected breast cancer cells is both safe and well tolerated. In addition to assessing safety, it is critically important to perform basic studies in order to determine if treatment resulted in the immunologic recognition of the tumor (even in the absence of a clinical response) and to dissect some of the mechanisms by which expression of B7 induces a host immune response in human breast cancer. Detecting an immune response would indicate that the theoretical basis for the strategy chosen was sound and this knowledge will lead us to continue our efforts to refine and improve the use of B7-transfected tumor cells.

Experimental Design.

Immunologic parameters to be assessed include (1) in vivo testing for delayed-type hypersensitivity reactions (DTH) to irradiated tumor cells; (2) biopsies of vaccination sites and draining lymph nodes for histologic and immunohistochemical assessment as well as for RT-PCR detection of cytokine gene expression; (3) determination of peripheral blood T cell subsets; (4) examination of serum for the development of anti-tumor antibodies; (5) determination of in vitro responses of peripheral blood T cells and cells from regional lymph nodes to cryopreserved tumor cells as assessed by proliferation, cytokine production, and tumor cell lysis. The cryopreserved tumor cells to be used for these studies will be aliquots of the same B7-transfected and non-transfected cells which will be used for vaccination. However, the cryopreserved tumor cells will not be irradiated prior to freezing. Testing will be performed at the following time points:

	Pretx	2 wks	4 wks	8 wks	12 wks
DTH			X	X	X
Skin & LN Biopsies		X			
T cell subsets	X	X	X	X	X
Antibody screening	X	X	X	X	X
In vitro T cell assays	X	X	X	X	X

Experimental Methods.

A. DETERMINATION OF T CELL SUBSETS.

Peripheral blood will be obtained by phlebotomy and mononuclear cells will be isolated by Ficoll-Hypaque density gradient centrifugation. Directly labeled monoclonal antibodies to human CD3, CD4, CD8, CD28, CD45RA, and CD45RO will be used for multi-color flow cytometry using various combinations of the commercially available antibodies conjugated to FITC, phycoerythrin, and PerCP. Cells will be stained using standard protocols and will be analyzed on a FACScan.

B. ANTIBODY SCREENING.

Serum samples from peripheral blood will be tested for antibodies directed against autologous B7-transduced and non-transduced breast cancer cells. Pre-treatment and post-treatment sera will be obtained as specified above, and stored at -80°C prior to assay, so that all samples from a given patient can be assayed simultaneously. Samples will be tested in serial dilutions for determination of antibody titer. The samples will be incubated with autologous B7-transduced or non-transduced breast cancer cells for 60 min on ice, washed three times, incubated with FITC-conjugated goat anti-human Ig, washed again, and analyzed by flow cytometry. Specificity of binding to breast cancer cells will be determined by screening serum against autologous T cells. To determine whether antibodies, if detected, are induced against only $B7^+$ cells, we will compare the results seen with $B7^+$ breast cancer cells and with non-transduced breast cancer cells. To determine if antibodies are directed at B7 itself, we will screen sera against $B7^+$ and $B7^-$ CHO cells. In positive serum samples, Ig isotype (IgM vs. IgG) will be determined by pre-treatment of samples with DTT to inactivate IgM antibodies.

C. BIOPSIES.

Biopsies of vaccination sites and of draining lymph nodes will be performed by Dr. Alfred Chang. Tissue samples will be processed for routine histologic staining to determine the presence of breast cancer cells and infiltrating lymphocytes or other inflammatory cells. Regional lymph nodes will be examined for evidence of immune responsiveness as assessed by hyperplasia. In addition, immunohistochemical staining will be performed for CD3, CD4, CD8, CD16 and CD28, to determine the phenotype of cells infiltrating the vaccination sites and draining lymph nodes and for HLA-class II and IL-2R to determine the activational status of these cells. In the case of skin biopsies, we will also stain with anti-CD1a to determine whether Langerhans cells have been recruited into the injection site and for HLA-class I and II expression on tumor cells. Dr. Brian Nickoloff, who has extensive experience in the clinical and *in vitro* analysis of immunologic responses occurring in the skin will evaluate these tissue specimens. Specimens to be examined by immunofluorescent staining will be frozen in O.C.T. embedding compound, sectioned and stained for T cell subsets. Single color staining will be done by immunoperoxidase, as this will permit simultaneous evaluation of tissue histology. Two color staining will be accomplished with one antibody labeled with fluorescein, and the second labeled with biotin followed by streptavidin-Texas Red.

D. RT-PCR.

If the biopsy of the vaccination site contains infiltrating lymphocytes, we will use semi-quantitative reverse transcriptase-assisted PCR to determine whether cytokine mRNAs are present in

biopsy sites. Cytokines to be studied will be IL-2, IL-4, IL-10, IL-12, GM-CSF, and IFNγ. RNA will be isolated using acid-phenol. Total RNA samples (1-5 μg) will be incubated for 10 min at 65° C, cooled for 3 min on ice, and reverse-transcribed into cDNA. The reverse transcription reactions will be then heat inactivated 95° C for 10 min and cooled for 3 min. The primers to be used for amplification of IL-2, IL-4, IL-10, and IFNγ, and the cycling conditions, have been previously described (84) and used by our laboratory for detection of cytokine mRNAs (Naidu et al., submitted). The IL-12 primers were synthesized based on published IL-12 cDNA sequence, and have been verified to be specific for IL-12 based on predicted size and specific hybridization with an internal oligonucleotide (Goodman et al., unpublished data). To ensure that differences in cytokine product amount correlate with differences in starting cDNA (and hence mRNA) amount, we will ensure that PCR amplification is in the exponential phase. This will be done by verifying that the intensity of the product bands (both cytokines and controls) is increasing for at least 3 consecutive PCR cycles and that their relative ratios are constant. After amplification, the PCR products will be resolved by electrophoresis in a 1.5% agarose gel blotted on a nylon membrane and hybridized to ^{32}P-labeled internal oligonucleotide probes (sequences indicated above). The membranes will then be analyzed on a phosphor-imager for accurate measurement of bound radioactivity. All reverse-transcribed RNA samples will be simultaneously amplified using β-actin and/or GAPDH primers to verify that the RNA was intact and that reverse transcription was successful. This will also permit the calculation of cytokine:control mRNA ratios for normalization between samples.

E. DTH.

Patients will be tested for DTH responses by intradermal injections of both irradiated autologous B7-transfected breast cancer cells and irradiated autologous nontransfected and vector-alone transfected breast cancer cells at separate sites on the volar surfaces of both forearms. There will be three separate injections of 10^3, 10^4, and 10^5 cells on both forearms. Induration will be assessed 24 and 48 hours later. DTH responses to all three doses of autologous tumor cells will be measured and recorded as the largest biperpendicular diameters of induration at 24, 48, and 72 hour time points. A positive DTH test will be defined as induration measuring greater than 25 mm^2 overall (determined by multiplying biperpendicular diameters) as measured at any of the three time points. Control DTH responses will be tested simultaneously using Candida and/or mumps antigen. Although patients known to be anergic are excluded from the protocol, this will verify that non-responsiveness to breast cancer cells is not part of a generalized anergic state developing during, or as a consequence of, treatment.

F. IN VITRO T CELL ASSAYS.

i. proliferation.

Mononuclear cells from peripheral blood or lymph nodes will be co-cultured with irradiated autologous B7-transfected and non-transfected tumor cells in 96-well tissue culture plates. Proliferation will be assessed by addition of ^{3}H-TdR (1 μCi/well) for the last 6 hours of a 6 day culture period. Negative control cultures will be wells containing mononuclear cells without tumor cells. Positive controls will be polyclonal mitogens such as anti-CD3 mAb, staphylococcal enterotoxin A and B, and PHA, and the duration of these cultures will be 72 hours. These will serve to verify that patients' lymphocytes are competent to respond to appropriate stimuli, and that lack of proliferation to tumor cells, if observed, is not part of a generalized immunosuppressive state. If a proliferative response to tumor cells is observed, we will determine whether or not CTLA4Ig and/or anti-B7 mAb (10 μg/ml) is capable of inhibiting this response. If so, this would indicate that this response is dependent on B7 expression in the relevant cells. Since these cultures will contain autologous antigen-presenting cells (known to express co-stimulatory signals such as B7), we will also isolate purified T cells from peripheral blood or lymph nodes and test their ability to proliferate to B7-transfected and non-transfected tumor cells. Our protocol using negative selection with monoclonal antibodies directed at B cells, macrophages, and NK cells (anti-CD14, anti-CD16, anti-CD19 plus anti-HLA DR) and magnetic beads is capable of producing highly purified (>99%) resting T cells devoid of functional accessory cells (85). The ability of purified T cells to respond to B7-transfected tumor cells would indicate that the tumor cell by itself is capable of inducing a T cell response, rather than providing a soluble neo-antigen which is presented by a B7$^+$ antigen-presenting cell. If purified T cells from patients vaccinated with B7-transfected tumor respond to non-transfected tumor cells, this would indicate that T cell activation in vivo by B7-expressing cells has led to the development of primed T cells which no longer require co-stimulation by B7 for their response.

ii. cytokine production.

Cultures conditions will be identical to those described above for proliferation studies. Culture supernatants will be frozen at -80°C for subsequent assay by ELISA for IL-2, IFNγ, IL-4, GM-CSF, and IL-10 using commercially available kits.

iii. tumor-cell lysis.

The ability of lymphocytes to lyse B7-transfected and non-transfected tumor cells will be ascertained by ^{51}Cr release. Briefly, tumor cells will be labeled with ^{51}Cr, and labeled target cells will be plated in 96-well round bottomed plates at 5000 cells/well. Varying number of washed effector cells ($0.25 - 0.5 \times 10^6$) will be added and will constitute the experimental well. The maximum or spontaneous release of ^{51}Cr will be determined by the addition of detergent, or of medium without effector cells, respectively. After 3.5 hours culture at 37°C, plates will be centrifuged and 0.1 ml of supernatant and counted in a gamma counter. Percent specific lysis is calculated as:

$$\frac{(\text{experimental cpm} - \text{spontaneous cpm}) \times 100}{(\text{maximum cpm} - \text{spontaneous cpm})}$$

In these assays both B7-transfected and control-transfected tumor targets will be used as well as the NK target K562. When responses are observed, we will determine the ability of anti-CD4, anti-CD8, anti-HLA class I and anti-B7 mAbs to block the response. Initially, we will test T cells obtained immediately after harvest for cytotoxicity. If we fail to see any response, we will test cells which have been restimulated in vitro by 7-day co-culture with B7-transfected or control-transfected tumor cells.

V. Investigator's Qualifications

John W. Smith II, M.D., Principal Investigator

Dr. Smith is currently an Associate Professor in the Department of Internal Medicine, Division of Hematology/Oncology. For the last seven years, he has conducted clinical research at the Biological Response Modifiers Program of the National Cancer Institute using a variety of immunologic treatments for cancer. He conducted definitive phase I and II clinical trials of interleukin-1. He was also involved in the design and conduct of a clinical trial of autologous renal cell carcinoma vaccine combined with IL-2 (Robert Fenton, M.D., Ph.D., Principal Investigator) and participated in the planning stages of an intramural protocol using a B7-transfected allogeneic melanoma cell line to immunize melanoma patients (Mario Sznol, M.D., Principal Investigator). Dr. Smith currently evaluates and treats breast cancer patients as a clinical researcher involved in the Breast Oncology Program. His extensive previous experience conducting phase I trials of biologicals makes him extremely well qualified to be in charge of all phases of the clinical protocol.

Laurence A. Turka, M.D., Associate Investigator

Dr. Turka is currently an Associate Professor in the Department of Internal Medicine. He also serves as the Director of the Immunobiology and Tumor Immunology Program of the University of Michigan Cancer Center. Dr. Turka's laboratory focuses on mechanisms of T cell development and T cell activation. In particular, much of his work over the past several years has focused on the CD28:B7 pathway of T cell activation. He has extensive experience in assays of lymphocyte function including proliferation, cytotoxicity, and lymphokine gene expression. Along with Dr. Alfred Chang, will oversee all aspects of this project related to immunological monitoring of patients, and studying in vitro immunogenicity of B7-transfected and control-transfected tumor cells.

Stephen P. Ethier, Ph.D., Associate Investigator

Dr. Ethier is currently an Assistant Professor in the Department of Radiation Oncology, Division of Cancer Biology at The University of Michigan Medical Center. Dr. Ethier has been working in the field of breast cancer research for over 13 years. Over the past five years, the work in Dr. Ethier's laboratory has focused on understanding cellular mechanisms of growth regulation in normal and neoplastic mammary epithelial cells. As part of his work, many new methods have been developed to allow isolation and growth of human breast cancer cells from patient biopsy specimens. This expertise makes him uniquely qualified to coordinate the preparation of the autologous tumor cell vaccines to be used in the clinical trial.

Alfred E. Chang, M.D., Associate Investigator

Dr. Chang is Professor of Surgery and Chief, Division of Surgical Oncology. He has been a leading investigator in the area of adoptive immunotherapy at both the experimental and clinical levels. As Associate Director for Clinical Affairs of the University of Michigan Cancer Center, he will be able to help identify and recruit appropriate candidates for this study from the clinicians of the Breast Oncology Program. He is actively involved in the diagnosis and treatment of patients with breast cancer at the University of Michigan. Dr. Chang has had extensive experience in the treatment of cancer patients with IL-2 and activated lymphocytes (i.e., LAK, TIL, IVS and anti-CD3/IL-2 activated cells). His vast prior clinical and laboratory experience in immunotherapeutic approaches to the treatment of cancer makes him imminently qualified to be responsible for the surgical and immunological monitoring aspects of this trial.

Barbara Weber, M.D., Collaborator

Dr. Weber is an Assistant Professor in the Department of Internal Medicine, Division of Hematology/Oncology. She is a well-trained molecular geneticist and a recognized expert in the field of breast cancer genetics. She is now the Director of the Breast Oncology Program for the University of Michigan Comprehensive Cancer Center. Her laboratory has been in the forefront of the search for BRCA1 since 1991, when this gene was identified by genetic linkage analysis. In addition, she has direct patient care responsibilities for over 400 breast cancer patients and will be able to identify potential patients for enrollment onto this trial and will encourage the clinicians involved in the Program to consider this study as an option for appropriate patients.

Brian J. Nickoloff, M.D., Ph.D., Collaborator

Dr. Nickoloff is an Associate Professor of Pathology and Dermatology. He has extensive experience in the histological and immunohistochemical evaluation of skin biopsies including the use of two-color immunohistochemistry.

F. Statement of Work

Task 1: Develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer. Months 1-30.

a. Patients with metastatic or locally recurrent breast cancer will be identified.
b. Metastatic tumor will be surgically removed from patients identified in "a".
c. Enzymatic and mechanical dissociation methods will be used to obtain single cell suspensions.
d. A series of monoclonal antibodies will tested using immunomagnetic beads in order to isolate a highly purified population of breast cancer cells.

Task 2: Study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA. Months 1-30.

a. Determine optimal methods of adenoviral transduction of breast cancer cells using an adenoviral-LacZ vector.
b. Assess efficiency and longevity of LacZ expression.
c. Use the methods developed for adenoviral-LacZ to transfect breast cancer cells with an adenoviral-B7 vector.
d. Assess efficiency and longevity of B7 expression in irradiated and non-irradiated cells.
e. Assess ability of irradiated and non-irradiated B7-expression breast cancer cells to provide co-stimulatory signals for autologous T cells.

Task 3: Conduct a Phase I clinical trial of B7 transfected breast cancer cells as a vaccine. Months 1-48, minimum of 26 patients will be evaluated.

a. Identify eligible breast cancer patients.
b. Obtain a source of autologous breast cancer cells by thoracentesis, paracentesis or surgical excision of skin or lymph node metastases, as appropriate.
c. Transduce the isolated tumor cells with the human B7 gene.
d. Radiate the transfected tumor cells and then vaccinate patients in the thigh.
e. Monitor patients closely for side effects and toxicities.
f. Evaluate patients for any evidence of tumor response.

Task 4: Perform in vitro and in vivo immunologic monitoring studies on enrolled patients to assess the development of an anti-tumor immune response. Months 3-48, in vitro analysis will be performed on all patients.

a. Skin biopsies will be performed at the site of tumor cell vaccination for routine histology, immunohistochemistry, and RT-PCR assessment of cytokine production by infiltrating lymphocytes (if found).
b. Enrolled patients will be assessed for delayed-type hypersensitivity response to autologous $B7^+$ and $B7^-$ tumor cells.
c. PBLs and T cells will be isolated from serial blood samples and from regional lymph nodes and cryopreserved. These cells will be tested for in vitro proliferative responses, cytokine production and lytic capacity using B7-transduced and control-transduced autologous tumor cells as stimulators/targets.
d. Serum samples from peripheral blood will be tested for antibodies directed against autologous $B7^+$ and control-transduced breast cancer cells.

COST ESTIMATE SUMMARY

	1YR	2ND	3RD	4TH	
SUBTOTAL	$138,545	$142,683	$146,947	$122,863	$551,038
INDIRECT	$62,414	59,366	61,583	63,889	$247,252
TOTAL	$200,959	202,049	208,530	186,752	$798,290

INSTITUTION: University of Michigan

AGREEMENT DATE: November 18, 1992

SECTION II: GENERAL

A. LIMITATIONS: The rate(s) in this Agreement is subject to any statutory or administrative limitations and apply to a given grant, contract, or other agreement only to the extent that funds are available. Acceptance of the rate(s) is subject to the following conditions: (1) Only costs incurred by the organization were included in its indirect cost pool as finally accepted; such costs are legal obligations of the organization and are allowable under the governing costs principles. (2) The same costs that have been treated as indirect costs are not claimed as direct costs. (3) Similar type of costs have been accorded consistent accounting treatment. (4) The information provided by the organization which was used to establish the rate(s) is not later found to be materially incomplete or inaccurate.

B. ACCOUNTING CHANGES: If a fixed or predetermined rate(s) is contained in this Agreement, it is based on the accounting system in effect at the time the agreement was negotiated. Changes to the method of accounting for costs which affect the amount of reimbursement resulting from the use of this Agreement require prior approval of the authorized representative of the cognizant agency. Such changes include, but are not limited to, changes in the charging of a particular type of costs from indirect to direct. Failure to obtain such approval may result in costs disallowances.

C. FIXED RATES: If a fixed rate(s) is contained in this Agreement, it is based on an estimate of the costs for the period covered by the rate(s). When the actual costs for this period are determined, an adjustment will be made in a subsequent Agreement to compensate for the difference between the costs used to establish the fixed rate(s) and actual costs.

D. USE BY OTHER FEDERAL AGENCIES: The rate(s) in this Agreement is approved in accordance with the authority in Office of Management and Budget Circular A-[illegible] and should be applied to grants, contracts and other agreements covered by Office of Budget and Management Circular A-21, subject to any limitations in A above. The organization may provide copies of this Agreement to other Federal Agencies to give them early notification of the Agreement.

E. OTHER: If any Federal contract, grant or other agreement is reimbursing indirect costs by a means other than the approved rate(s) in this agreement, the organization should (1) credit such costs to the affected programs and (2) apply the approved rate(s) to the appropriate base to identify the proper amount of indirect costs allocable to these programs.

INSTITUTION: University Of Michigan

AGREEMENT DATE: November 18, 1992

F. SPECIAL REMARKS: SEE ATTACHMENT

DEFINITION OF OFF-CAMPUS RATES:
A project shall be considered an off-campus activity if more than 50% of the direct salaries and wages of personnel engaged on the project are incurred at a site neither owned nor leased by the University.

The University's policy provides that an item of nonexpendable tangible personal property having a useful life of two years or more and an acquisition cost of $500 or more is classified as equipment and is capitalized.

TREATMENT OF PAID ABSENCES:
Vacation, holiday, sick leave pay and other paid absences are included in salaries and wages and are claimed on grants, contracts and other agreements as part of the normal cost for salaries and wages. Separate claims for the costs of these paid absences are not made.

BY THE ORGANIZATION:
University of Michigan

(ORGANIZATION)

CW Matthews

(SIGNATURE)

C. W. Matthews
(NAME)

Associate V.P. for Finance
(TITLE)

November 20, 1992
(DATE)

BY THE COGNIZANT AGENCY
ON BEHALF OF THE FEDERAL GOVERNMENT:
DEPT OF HEALTH AND HUMAN SERVICES

(AGENCY)

(SIGNATURE)

for Kenneth R. Gibbons
(NAME)

Director, Division of Cost Allocation
(TITLE)

November 18, 1992
(DATE) 5064

HHS REPRESENTATIVE: Robert J. Vernon
Telephone: (312) 353-9315

RATE AGREEMENT
COLLEGES AND UNIVERSITIES

EIN# 1-386006309-A1

DATE: November 18, 1992

INSTITUTION:
University of Michigan
Ann Arbor, Michigan 48109

FILING REF: The preceding Agreement was dated May 20, 1992

The rates approved in this Agreement are for use on grants, contracts and other agreements with the Federal Government subject to the conditions in Section II.

SECTION I: RATES

Type	Effective Period From	To	Rate	Locations	Applicable To
INDIRECT COST RATES*					
Predetermined	7/1/92	6/30/93	55.8(1)	On-Campus	Research(A)
Predetermined	7/1/92	6/30/93	29.5(1)	Off-Campus	Research(A)
Predetermined	7/1/92	6/30/93	25.0(1)	Vessel	Research(A)
Predetermined	7/1/92	6/30/93	72.0(1)	On-Campus	Instruction(A)
Predetermined	7/1/92	6/30/93	32.5(1)	On-Campus	OSA(A)

(A) APPLICABLE TO ALL AWARDS EFFECTIVE PRIOR TO 7/1/92.
(B) APPLICABLE TO ALL AWARDS EFFECTIVE ON OR AFTER 7/1/92.

The rates in this agreement have been negotiated or revised, as appropriate, to reflect the administrative cap provisions of the revision to the OMB Circular A-21 published by the Office of Management and Budget on October 3, 1991. No rate affecting the institution's fiscal periods beginning on or after October 1, 1991 contains total administrative cost components in excess of that 26 percent cap.

*BASE:
(1) Total direct cost to include tuition remissions paid in lieu of wages but excluding capital expenditures (alterations, improvements, renovations, equipment-See Special Remarks), the portion of each individual subgrant or subcontract in excess of $10,000, patient care charges, and the cost of services provided by the University's Computing Center, the Unit for Lab Animal Medicine, the Michigan Memorial Phoenix Laboratories and Vessel Services.

(2) Total direct cost excluding capital expenditures (alterations, improvements, renovations, equipment-See Special Remarks), the portion of each individual subgrant or subcontract in excess of $10,000, patient care charges, student tuition remission and student support costs (e,g., student aid, stipends, dependency allowances, scholarships, fellowships), the cost of services provided by the University's Computing Center, the Unit for Lab Animal Medicine, the Michigan Memorial Phoenix Laboratories and Vessel Services.

TREATMENT OF FRINGE BENEFITS:
Fringe benefits applicable to direct salaries and wages are treated as direct costs.

INSTITUTION: University of Michigan

AGREEMENT DATE: November 18, 1992

SECTION I: RATES (CONTINUED)

Type	Effective Period From	To	Rate	Locations	
Applicable To					
INDIRECT COST RATES*					
Predetermined	7/1/92	6/30/93	52.0(1)	On-Campus	Research(B)
Predetermined	7/1/92	6/30/93	26.0(1)	Off-Campus	Research(B)
Predetermined	7/1/92	6/30/93	25.0(1)	Vessel	Research(B)
Predetermined	7/1/92	6/30/93	72.0(1)	On-Campus	Instruction(B)
Predetermined	7/1/92	6/30/93	32.5(1)	On-Campus	OSA(B)
Predetermined	7/1/93	6/30/95	52.0(2)	On-Campus	Research(B)
Predetermined	7/1/93	6/30/95	26.0(2)	Off-Campus	Research(B)
Predetermined	7/1/93	6/30/95	25.0(2)	Vessel	Research(B)
Predetermined	7/1/93	6/30/95	72.0(2)	On-Campus	Instruction(B)
Predetermined	7/1/93	6/30/95	32.5(2)	On-Campus	OSA(B)
Provisional	7/1/95	6/30/96	Use the same rates as in effect for the year ended June 30, 1995.		

SENIOR PERSONNEL	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
John W. Smith II, M.D.	Principal Investigator	12	10	$84,350	$8,435	$1,771	$10,206
Laurence Turka, M.D.	Associate Investigator	12	10	$109,102	$10,910	$2,291	$13,201
Stephen Ethier, Ph.D.	Associate Investigator	12	10	$69,595	$6,960	$1,462	$8,422
Alfred Chang, M.D.	Associate Investigator	12	5	$124,375	$6,219	$1,306	$7,525
Barbara Weber, M.D.	Collaborator	12	0	$0	$0	$0	$0
Brian J. Nickoloff	Collaborator	12	0	$0	$0	$0	$0
	SUBTOTALS——>				$32,524	$6,830	$39,354

OTHER PERSONNEL	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
TBA	Research Assistant I	12	50	$22,000	$11,000	$2,860	$13,860
TBA	Research Assistant II	12	50	$22,000	$11,000	$2,860	$13,860
Pam Boughan	Data Manager	12	30	$26,000	$7,800	$2,028	$9,828
TBA	Clinical Nurse	12	15	$46,000	$6,900	$1,794	$8,694
Amy Laura	Research Secretary III	12	5	$22,335	$1,117	$290	$1,407
Julie Goldstein	Grant Manager	12	5	$24,570	$1,229	$320	$1,549
	SUBTOTALS——>				$39,046	$10,152	$49,198

PERSONNEL TOTAL **$88,552**

SUPPLIES

Enzymes	$2,000	
Tissue Culture	$6,000	
Radioisotopes	$1,000	
ELISA kit	$2,000	
Monoclonal Antibodies	$1,000	
Chemicals	$1,500	
Plasticware	$2,000	
Immunological reagents	$4,000	
		$19,500

TRAVEL

Travel for PI to travel to the International Conference on
Gene Therapy of Cancer, San Diego, CA in yrs 1,2, and 3 to present data & remain current
Will attend in year 4 USARDC conference.

400 round trip airfare	400	
375 for registration/ 450 per diem	825	$1,225

OTHER EXPENSES

Publication costs: $40 per page @ 10	400	
Postage	100	
Telephone and xerox cost	250	
		$750

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$110,027
CONSORTIUM/CONTRACTUAL COSTS		
DIRECT COSTS	17,442	
INDIRECT COSTS	11,076	28,518
Total Directs		**$138,545**
Indirects		$62,414
GRAND TOTAL		**$200,959**

	1YR	2ND	3RD	4TH	
PERSONNEL	$88,552	91,831	95,202	98,707	
SUPPLIES	$19,500	$20,280	$21,091	$21,935	
TRAVEL	$1,225	1,274	1,325	1,378	
OTHER EXP	$750	780	811	843	
CONTRACT	$28,518	$28,518	$28,518	0	
SUBTOTAL	$138,545	$142,683	$146,947	$122,863	$551,038
INDIRECT	$62,414	59,366	61,583	63,889	$247,252
TOTAL	$200,959	202,049	208,530	186,752	$798,290

Budget Justification

Tissue Culture Supplies ($6,000): These consist of media (RPMI-1640), media supplements, fetal calf serum, disposable pipets and pipet tips, plasticware (tissue culture flasks, 96-well plates). We would note in particular the high cost of fetal calf serum ($150/500 ml bottle).

Fetal Calf Serum (10 bottles/year)	$2000
Media and media supplements	1000
Plasticware	3000
Total	$6000

Radioisotopes ($1000): 3H-thymidine will be used for proliferation assays; 51Cr-sodium chromate will be used for cytotoxicity assays, and 32P-dCTP will be used for semiquantitative RT-PCR assessment of in vivo cytokine gene expression.

ELISA kits ($2000): Cytokine gene expression in tissue culture will be determined by ELISA. Commercially available kits from TAGO are $500/kit which allows one to run 2 complete 96-well microtiter plates/kit (total of 192 wells). By running several samples simultaneously to avoid replicating standards, we anticipate that the purchase of 4 kits/year will suffice.

Monoclonal Antibodies ($1000): Directly labelled mAbs will be purchased for flow cytometry and immunohistochemistry. These often cost $200-400/vial, although each vial can be used for up to 100 test samples and may have a shelf life of 2-3 years. Averaged over the time course of this grant, $1000/year should provide sufficient reagents for these studies.

Chemicals ($1500): These include primarily reagents for RT-PCR such as restriction enzymes, buffers, DNA modifying enzymes, Taq polymerase etc, and miscellaneous other chemicals.

Immunological reagents ($4000): Will be used for cell isolation and characterization.

Enzymes ($2000): Will be used for tissue dissociation.

Other: Funds are requested to cover costs of publications, postage, telephones, and xeroxing.

SUBCONTRACT

CERTIFICATION OF RECOMBINANT ADENOVIRAL REAGENTS

Establishment and Preservation of the 293 Master Cell Bank (1/5 cost based on 5 users)	12,846
Certification of Recombinant Seed Lot	8,330
Certification and Characterization of Cell Lysate	470
Certification of Recombinant Adenoviral Production Lot	1,780
Sequencing of Adenoviral DNA	10,000
	$33,426
Manufacture of 2 recombinant adenoviral production lots (includes steps #3 and #4)	8,900
	$42,326
Manufacturing Supplies ($5,000.00 per lot x 2 lots)	10,000
Grand Total Direct = 2 lots	$52,326
Indirects @ 63.5%	33,227
The total costs for 2 lots will be spread over 3 years of the project, to coincide with duration of clinical trial.	$28,518

Budget Justification

Personnel

John W. Smith II, M.D., Principal Investigator: Dr. Smith will be responsible for all aspects of the conduct of the clinical trial in this grant proposal including: patient evaluation, review of eligibility criteria, vaccination of patients with B7 transfected tumor cells, and close clinical monitoring for toxicities. He will supervise the research nurse and data manager involved in this study. It is anticipated that one half day per week will be sufficient to perform these duties, and therefore, 10% salary support is requested for this effort.

Laurence A. Turka, M.D., Co-Investigator: Dr. Turka will be responsible for the immunological monitoring studies to be performed including DTH testing, studies of proliferation, cytokine release and tumor cell killing, and assays of antibody production. He, along with Dr. Nickoloff (consultant) will evaluate biopsies of vaccination sites by immunohistochemistry and RT-PCR. This is anticipated to require 4 hours per week and therefore 10% effort is requested.

Alfred Chang, M.D., Co-Investigator: Dr. Chang will perform all the biopsies of the vaccination sites and all the lymph node excisions on the patients in this trial. Dr. Chang will work with Dr. Turka on cellular assays in the immunological monitoring studies. This is anticipated to require 2 hours per week and therefore 5% effort is requested.

Stephen Ethier, Ph.D., Co-Investigator: Dr. Ethier will also be responsible for all of the experiments aimed at developing methods for selective isolation of human breast cancer cells from patient tumor samples. Dr. Ethier will also directly supervise experiments to optimize expression of transduced genes in breast cancer cells using adenoviral expression vectors containing both the Lac Z gene and human B7 gene. Finally, Dr. Ethier will oversee the aspect of the project aimed at preparing human breast cancer cells expressing the B7 gene for the preparation of the tumor cell vaccines to be used in the Phase I clinical trial. 10% effort is committed to this project.

Radiation Oncology Technician: To carry out these experiments one, half-time, research assistant will work under the direction of Dr. Ethier. 50% effort is requested.

Research Assistant II: This individual will perform all of the above studies under the guidance of Drs. Turka and Chang. It is anticipated that this will require 20 hours per week due to the large numbers of samples and tests to be done. 50% effort is requested.

Research Nurse (TBA): The research nurse involved in this clinical project will be involved in initial patient contact to screen for possible eligibility for the study, patient evaluation, patient care including monitoring the patient for toxicities and side effects, grading of such toxicities, and frequent follow-up contacts to assess the tolerability of treatment. It is anticipated that this will take 6 hours/week and therefore, 15% salary support is requested for this effort.

Pam Boughan, Data Manager: The data manager for this clinical trial will be involved in insuring that all necessary tests are obtained and documented. This includes filling in flow sheets with the appropriate information regarding treatment toxicities and laboratory studies. In conjunction with the research nurse, she will insure that tumor measurements are performed and recorded and that all laboratory data including the immunologic monitoring data are collected and recorded. Records will be maintained on paper as well as on a computer database and spreadsheet. It's anticipated that 1.5 days/week will be necessary to perform these tasks, therefore 30% salary support is requested.

Amy Laura, Research Secretary III: Ms. Laura works directly with the principal investigator and will provide general office and administrative support including preparation of manuscripts, facsimile transmissions, correspondence, travel, and telephone traffic related to this research support. It is anticipated that 2 hours/week will be necessary to perform these tasks, therefore 5% salary support is requested.

Julie Goldstein, Grant Manager: Ms. Goldstein will be involved in the accounting and financial management of the grant. She will be the contact for the institution, the P.I. and the USAMRDC for any questions regarding financial reporting. This effort will take 2 hours/week, therefore 5% salary support is requested.

Salary for all individuals is increased in years 2-4 by a 4% cost of living adjustment.

There is no negotiated fringe benefit rate at the University of Michigan. The rates used in this proposal, 21% for faculty and 26% for others, are derived from the experience rate, and represent an estimate. Components of the fringe benefit package may include health care (for individual and family members), long term disability, life insurance, dental and retirement, and varies depending on the complement of plans in which the individual is enrolled.

The subcontract in this proposal is a fixed price agreement for a supply of recombinant adenovirus expressing human B7. The subcontractors are James M. Wilson, M.D., Ph.D., Director of the Institute for Human Gene Therapy, University of Pennsylvania and his colleague, Stephen L. Eck, M.D., Ph.D. The subcontractors were selected in the absence of competition for the following reasons: 1) they had collaborated well with investigators at the University of Michigan in the past and were currently collaborating on other research projects; 2) Dr. Wilson is an international leader in gene therapy and techniques to prepare recombinant adenoviral vectors, and 3) they were preparing these vectors already and agreed to provide them on a cost basis thereby eliminating startup costs and markups resulting in substantial cost savings.

Acronym and Symbol Definition

SEER	Surveillance, Epidemiology and End Results
CTL	Cytotoxic T lymphocytes
APC	Antigen presenting cell
TCR	T cell antigen receptor
IL-	Interleukin-
IL-2R	IL-2 receptor
Th	T helper
CD	Cluster designation
mRNA	Messenger ribonucleic acid
kd	Kilodalton
mAb	Monoclonal antibody
IFNγ	Interferon-gamma
LPS	Lipopolysaccharide
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
PHA	Phytohemagglutinin
Ig	Immunoglobulin
nM	Nanomolar
MLC	Mixed lymphocyte culture
μg/ml	Microgram/milliliter
NK	Natural killer
LAK	Lymphokine activity killer
TIL	Tumor infiltrating lymphocyte
TNF	Tumor necrosis factor
GM-CSF	Granulocyte macrophage colony stimulating factor
MHC	Major histocompatability complex
HLA	Human leukocyte antigen
pfu	Plaque forming units
mg	Millogram
rpm	Revolutions per minute
g	Gravity
CMF	Calcium, magnesium free
BSS	Balanced salt solution
EDTA	Ethylenediaminetetraacetic
MPC	Magnetic particle concentrators
IRB	Institutional Review Board
RAC	Recombinant DNA Advisory Committee
FDA	Food and Drug Administration
DTH	Delayed type hypersensitivity
ANA	Antinuclear antibody
RF	Rheumatoid factor
CH50	Total complement
T4	Thyroxine
TSH	Thyroid stimulating hormone
DTT	Dithiothreitol
RT-PCR	Reverse transcriptase assisted polymerase chain reaction
FITC	Fluorescein isocyothionate
GAPDH	Glyceraldehyde phosphate dehydrogenase

Bibliography

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BIOGRAPHICAL SKETCH

NAME: John W. Smith II POSITION TITLE: Associate Professor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Amherst College, Amherst, MA	M.A.	1973	
University of Pennsylvania, Philadelphia, PA	B.A.	1976	
Jefferson Medical College, Philadelphia, PA	M.D.	1981	Medicine

Professional Experience:

1986-90 Senior Staff Fellow, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland

1990-93 Medical Officer, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland

1993- Associate Professor, University of Michigan Hospitals, Department of Internal Medicine, Division of Hematology/Oncology

Publications (selected from list of 38)

Urba WJ, Clark JW, Steis RG, Bookman MA, Smith II JW, Beckner S, Maluish AE, Rossio JL, Rager H, Ortaldo, JR and Longo DL: Intraperitoneal lymphokine-activated killer cell/interleukin-2 therapy in patients with intra-abdominal cancer: Immunologic considerations. J Natl Cancer Inst. 81:602-611, 1989.

Clark JW, Smith II JW, Steis RG, Urba WJ, Crum E, Miller R, McKnight J, Beman J, Stevenson HC, Creekmore S, Stewart M, Conlon K, Sznol M, Kremers P, Cohen P, and Longo DL. Interleukin-2 (IL-2) and lymphokine activated killer cell therapy: Analysis of a bolus IL-2 and a continuous infusion IL-2 regimen. Cancer Res. 50:7343-7350, 1990.

Smith II, JW, Steis RG. Phase I evaluation of recombinant tumor necrosis factor given in combination with recombinant interferon gamma. J Immunoth. 10:355-362, 1991.

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Smith II, JW, Urba WJ, Curti BD, Elwood L, Steis RG, Janik JE, Sharfman WH, Miller L, Fenton RG, Conlon, KC, Sznol M, Creekmore SP, Wells N, Ruscetti FW, Keller J, Hestdal K, Shimizu M, Rossio J, Alvord WG, Oppenheim JJ, Longo DL. The toxic and hematologic effects of interleukin-1 alpha administered in a phase I trial to patients with advanced malignancies. J Clin Oncol. 10:1141-1152, 1992.

Sznol M, Clark, JW, Smith II, JW, Steis RG, Urba WJ, Rubinstein LV, VanderMolen LA, Janik J, Sharfman WH, Fenton RG, Creekmore SP, Kremers P, Conlon K, Hursey J, Beveridge J, Longo DL. Pilot study of interleukin-2 and lymphokine-activated killer cells combined with immunomodulatory doses of chemotherapy and sequenced with interferon alfa-2a in patients with metastatic melanoma and renal cell carcinoma. J Natl Cancer Inst. 84:9329-937, 1992.

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Janik J, Kopp WC, Smith II, JW, Longo DL, Alvord G, Sharfman WH, Fenton RG, Sznol M, Steis RG, Creekmore SP, Ewel CH, Hursey J, Urba WJ. Dose-related immunologic effects of levamisole in patients with cancer. J Clin Oncol. 11:125-135, 1993.

Kopp WC, Smith II, JW, Ewel CH, Alvord WG, Main C, Guyre PM, Steis RG, Londo DL, Urba WJ. Immunomodulatory effects of interferon-γ in patients with metastatic malignant melanoma. J Immunother. 13:181-190, 1993.

Curti DB, Longo DL, Ochoa AC, Conlon KC, Smith II, JW, Alvord WG, Creekmore SP, Fenton RG, Gause BL, Holmlund J, Janik JE, Ochoa J, Rice PA, Sharfman WH, Sznol M, Urba WJ. Treatment of cancer patients with ex vivo anti-CD3-activated killer cells and interleukin-2. J Clin Oncol. 11:652-660, 1993.

No extended periods of leave such as sabbatical are planned during the time frame of this grant.
Proportion of time to be devoted to this research and to other research: 20%
Identification of current or prior Federal Government Service: Dr. Smith was employed by the National Cancer Institute, Biological Response Modifiers Program from 7/86 through 7/93.
Number of graduate students: None.

BIOGRAPHICAL SKETCH

NAME: Laurence A. Turka

POSITION TITLE: Associate Professor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Summa Cum Laude, Colgate Univ.	B.A.	1974-78	Biochemistry
Cum Laude, Yale Medical College	M.D.	1978-82	Medicine

Professional Experience:

1982-85	**Resident**, Department of Internal Medicine, Yale-New Haven Hospital, New Haven, CT
1985-88	**Fellow**, Division of Nephrology, Department of Internal Medicine, Brigham and Women's Hospital, Boston, MA
1988-93	**Assistant Professor**, Division of Nephrology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI
1993-	**Associate Professor**, Division of Nephrology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI

Bibliography:

Turka LA, Tanaka K, Kupiec-Weglinski JW, Milford EL, Tilney NL, and Carpenter CB. In vivo activity of mixed lymphocyte response-generated suppressor cells and ability to prolong cardiac allograft survival in rats. Transplantation 1989; 47:388.

Turka LA, Ledbetter JA, Lee K, June CH, and Thompson CB. CD28 is an inducible T cell surface antigen that t ransduces a proliferative signal in CD3+ mature thymocytes J. Immunol. 1990; 144:1646.

Turka LA, Linsley PS, Paine R III, Schieven GL, Thompson CB, and Ledbetter JA. Signal transduction via CD4, CD8 and CD28 in immature thymocytes: Implications for thymic selection. J. Immunol. 1991; 146:1428..

Vandenberghe P, Freeman GJ, Nadler LM, Fletcher MC, Kamoun M, Turka LA, Ledbetter JA, Thompson CB, and June CH. Antibody and B7/BB1-mediated ligation of the CD28 receptor induces tyrosine phosphorylation in human T cells. J. Exp. Med. 1992; 175:951-960.

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Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74:597-608.

Lin H, Bolling SF, Linsley PS, Wei R-Q, Gordon D, Thompson CB, Turka LA. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor specific transfusion. J. Exp. Med. (in press).

No extended periods of leave such as sabbatical are planned during the time frame of this grant.
Proportion of time to be devoted to this research and to other research: 25%
Identification of current or prior Federal Government service: None.
Number of graduate students: three post-doctoral fellows, however he is not currently responsible for any graduate students.

BIOGRAPHICAL SKETCH

NAME: Stephen P. Ethier

POSITION TITLE: Assistant Professor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
St. Anselms College, Manchester, NH	B.A.	1977	Biology
University of Michigan, Ann Arbor, MI	M.S.	1979	Radiological Health
University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences Oak Ridge, Tennessee	Ph.D.	1982	Radiation Biology/ Carcinogenesis

Professional Experience:

1982-84	Post-Doctoral Fellow, Department of Chemical Carcinogenesis, The Michigan Cancer Foundation
1984-85	Scientist, Department of Chemical Carcinogenesis, The Michigan Cancer Foundation
1985-87	Assistant Member, Department of Chemical Carcinogenesis, The Michigan Cancer Foundation, Detroit, MI
1987-88	Assistant Member, Breast Cancer Group, The Michigan Cancer Foundation, Detroit, MI
1988-	Assistant Professor, Department of Radiation Oncology, The University of Michigan Medical School
1990-	Member, University of Michigan Cancer Center

Publications:

Adams LM, Ethier SP and Ullrich RL. "Survival of Mammary Epithelial Cells from Virgin Female BALB/c mice Following In Vivo - Irradiation". Radiat Res 100:264-272, 1984.

Ethier SP. "Primary Culture and Serial Passage of Normal and Carcinogen Treated Rat Mammary Epithelial Cells In Vitro. J Natl Cancer Inst 74:1307-1318, 1985.

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Ethier SP, Kudla A, and Cundiff KC. "Importance of Hormone and Growth Factor Interaction on the Proliferative Potential of Normal Rat Mammary Epithelial Cells In Vitro". J Cell Physiol. 132:161-167. 1987.

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Ethier SP, Mahacek ML, Gullick WJ, Frank, TS, Weber BL. Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res. 53:627-635, 1993.

Ethier SP, and Taback E. Insulin-like growth factor I independence in rat mammary carcinoma cells: A dominant pheotyped in somatic cell hybridization experiments. Cancer Lett., in press.

Mahacek ML, Beer D, Frank TS and Ethier SP. Finite proliferative lifespan in vitro of a human breast cancer cell strain isolated from a metastatic lymph node. Breast Cancer Res and Treat, in press.

No extended periods of leave such as sabbatical are planned during the time frame of this grant.

Proportion of time to be devoted to this research and to other research: Percent effort 15%, Time Commitments: 100%.

Identification of current or prior Federal Government Service: None

Number of graduate students: None

BIOGRAPHICAL SKETCH

NAME: Alfred E. Chang, M.D.

POSITION TITLE: Professor of Surgery

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Dartmouth College, Hanover, NH	B.A.	1971	Chemistry
Harvard Medical School, Boston, MA	M.D.	1974	Medicine
Duke University Medical Center, Durham, NC		1974-79	Surg Int/Res
National Cancer Institute, Bethesda, MD		1976-79	Surg Onc
Hosp Univ of Penn, Philadelphia, PA		1979-82	Surg Res

Professional Experience:

1982-87	Senior Investigator, Surgery Branch, NCI, Bethesda, MD
1984-87	Assistant Professor of Surgery, Uniformed Services of the Health Sciences, Bethesda, MD
1988-	Director, GI Tumor Program, University of Michigan Cancer Center
1988-	Associate Director, Clinical Affairs, University of Michigan Cancer Center
1988-92	Associate Professor of Surgery, University of Michigan, Ann Arbor, MI
1992-	Professor of Surgery, University of Michigan, Ann Arbor, MI
1988-	Chief, Division of Surgical Oncology, University of Michigan, Ann Arbor, MI

Publications:

Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Seipp CA, Simpson C, Reichert CM: Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med. 313:1485-1492, 1985.

Lotze MT, Chang AE, Seipp CA, Simpson RN, Vetto JT, Rosenberg SA. High dose recombinant interleukin-2 in the treatment of patients with disseminated cancer: Responses, treatment related morbidity and histologic findings. JAMA. 256:3117-3124, 1986.

Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FD, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT, Seipp CA, Simpson CG, White DE: Clinical experience with the treatment of 157 patients with advanced cancer using lymphokine activated killer cells and interleukin-2 or high dose interleukin-2 alone. N Engl J Med. 316:889-897, 1987.

Chou T, Chang AE, and Shu S: Generation of therapeutic T lymphocytes from tumor-bearing mice by in vitro sensitization: culture requirements and characterization of immunologic specificity. J Immunol. 140:2453-2461, 1988.

Topalian SL, Solomon D, Avis FP, Chang AE, Freerksen DL, Linehan WM, Lotze MT, Robertson CN, Seipp CA, Simon P, Simpson CG, and Rosenberg SA: Immunotherapy of patients with advanced cancer using tumor infiltrating lymphocytes and recombinant interleukin-2: a pilot study. J Clin Onc. 6:839-853, 1988.

Chang AE, Perry-Lalley DM, Shu S: Distinct immunologic specificity of tumor regression mediated by effector cells isolated from immunized and tumor-bearing mice. Cell. Immunol. 120:419-429, 1989.

Yoshizawa H, Chang AE, and Shu S: Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. J Immuno. 147:729-737, 1991.

Yoshizawa H, Sakai K, Chang AE, and Shu S: Activation by anti-CD3 of tumor-draining lymph node cells for specific adoptive immunotherapy. Cell Immunol. 134:473-479, 1991.

Nabel GJ, Chang AE, Nabel EG, Plautz GE, Fox BA, Huang L, and Shu S: Immunotherapy of malignancy by in vivo gene transfer into tumors. Human Gene Therapy. 3:399-410, 1992.

Chang AE, Yoshizawa H, Sakai, K, Cameron MJ, Sondak VK, and Shu S: Clinical observations on adoptive immunotherapy with vaccine-primed T lymphocytes secondarily sensitized to tumor in vitro. Cancer Res. 53:1043-1050, 1993.

Nabel GJ, Nabel E, Yang Z, Fox B, Plautz G, Gao X, Huang L, Shu S, Gordon D, and Chang AE: Direct gene transfer for the treatment of human disease-A trial of immunotherapy for melanoma. Proc Natl Acad Sci (in press.

No extended periods of leave such as sabbatical are planned during the time frame of this grant.

Proportion of time to be devoted to this research and to other research: 45%
Identification of current or prior Federal Government service: None
Number of graduate students: None

BIOGRAPHICAL SKETCH

NAME: Barbara L. Weber, M.D.

POSITION TITLE: Assistant Professor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of Washington, Seattle, WA	B.S.	1978	Chemistry
University of Washington, Seattle, WA	M.S.	1982	Medicine

Professional Experience:

1982-85	Intern and Resident in Medicine, Yale - New Haven Hospital, New Haven, Ct.
1985-88	Fellow in Medical Oncology, Dana-Farber Cancer Institute, Boston, MA
1985-88	Clinical Fellow in Medicine, Harvard Medical School, Boston, MA
1988-90	Instructor in Medicine, University of Michigan, Ann Arbor, MI
1990-present	Assistant Professor in Medicine, University of Michigan, Ann Arbor, MI

Publications:

1 Biesecker, B.B., Boehnke, M., Calzone, K., Markel, D.S., Garber, J.E., Collins, F.S., Weber, B.L.: Genetic Counseling for Families with Inherited Susceptibility to Breast and Ovarian Cancer. J Am Med Assoc 1993;269:1970-1974.

2. Chamberlain, J.S., Boehnke, M., Frank, T.S., Kiousis, S., Xu, J., Guo, S.-W., Hauser, E.R., Norum, R.A., Helmbold, E.A., Markel, D., Keshavarzi, S.M., Jackson, C.E., Calzone, K., Garber, J., Collins, F.S., and Weber, B.L.: BRCA1 Maps Proximal to D17S579 on Chromosome 17q21 by Genetic Analysis. Am J Hum Genet 1993;52:792-798.

3. Flejter, W.L., Watkins, M., Abel, K.J., Chandrasekharappa, S.C., Weber, B.L., Collins, F.S., Glover, T.W.: Isolation and characterization of somatic cell hybrids with breakpoints spanning 17q21.3-q24 by FISH and PCR. Ctyogenet & Cell Genet. 1993;64:222-223.

4. Abel, K.J., Boehnke, M., Prahalad, M., Ho, P., Flejter, W.L., Watkins, M., VanderStoep, J., Chandrasekharappa, S., Collins, F.S., Glover, T.S., Weber, B.L.: A Radiation Hybrid Map of the BRCA1 Region of Chromosome 17q12-21. Genomics, 1993;17:632-641.

5. Weber, B.L., and Garber, J.E.: Familial Breast Cancer: Probabilities and Possibilities. JAMA 1993;270:1602-1603.

6. Weber, B.L., Horiguchi, J., Luebbers, R.L. and Kufe, D.W.: Post-transcriptional Stabilization of c-fms mRNA by a Labile Protein during Human Monocytic Differentiation. Mol. Cell. Biol 1989;9:769-775.

7. Sherman, M.L., Weber, B.L. Datta, R., Kufe, D.W.: Transcriptional and Posttranscriptional Regulation of CSF-1 by Tumor Necrosis Factor: Apparent Involvement of Arachidonic Acid Metabolites. J. Clin. Invest. 1990; 85(2):442-447.

8. Weber, B.L., Westin, E., Clarke, M.F.: Differentiation of Mouse Erythroleukemia Cells Enhanced by Alternately Spliced c-myb mRNA. Science 1990;249:1291-1293.

9. Danish, R., El-Awar, O., Langmore, J., Weber, B., Turka, L.A., Clarke, M.F.: C-myb effects on kinetic events during MEL cell differentiation.. Oncogene 1992;7:901-907.

10. Ethier, S.P., Mahacek, M.L., Gullick, W.J., Frank, T.S., Weber, B.L.: Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Research 1993;53:627-635.

No sabbaticals or extended leaves are anticipated.

Proportion of time to be devoted to this research and to other research: 80%

Identification of current or prior Federal Government service: None.

Number of graduate students: None.

Assistants: None.

Other Personnel: None

BIOGRAPHICAL SKETCH

NAME: Brian J. Nickoloff, M.D., Ph.D. POSITION TITLE: Associate Professor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Kalamazoo College, Kalamazoo, MI	B.A.	1974	Biology
Wayne State University School of Medicine	M.D.	1979	Medicine
Wayne State University School of Medicine Department of Biochemistry, Detroit, MI	Ph.D.	1983	Biochemistry

Professional Experience:

1974-76	Graduate Assistantship, Department of Biochemistry, Wayne State University
1979-80	Internship, Department of Internal Medicine, Harbor General-UCLA Hospital
1980-81	Resident in Pathology, University of California at San Diego
1981-83	Resident in Pathology, Brigham and Women's Hospital, Boston, Massachusetts
1983-84	Dermatopathology Fellow in Dermatology, Stanford University School of Medicine
1984-86	Assistant Professor of Dermatology, Stanford University School of Medicine
1986-87	Assistant Professor of Pathology, Stanford University School of Medicine
1987-89	Assistant Professor of Pathology & Dermatology, University of Michigan

Publications:

Griffiths C, Voorhees JJ, Nickoloff BJ: Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: Modulation by recumbinant gamma interferon and tumor necrosis factor. J Am Acad Dermatol 1989;20:617-629.

Nickoloff BJ, Griffiths C: T lymphocytes and monocytes bind to keratinocytes in frozen sections of normal skin biopsies maintained in organ culture in the presence of gamma interferon via lymphocyte function associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) interaction. J Am Acad Dermatol 1989;20:736-743.

Griffiths C, Esmann J, Fisher GJ, Voorhees JJ, Nickoloff BJ: Gamma interferon differentially modulated keratinocyte expression of HLA-DR and intercellular adhesion molecule-1 (ICAM-1): Evidence for a protein kinase C-dependent and independent pathway. Br J Dermatol 1990;122:333-342.

Barker J, Sarma V, Dixit V, Nickoloff BJ: Marked synergism between tumor necrosis factor-alpha and interferon-gamma in regulation of keratinocyte derived chemotactic and adhesion molecules. J Clin Invest 1990;85:605-608.

Barker J, Mitra RS, Griffiths C, Dixit V, Nickoloff BJ: Keratinocytes as initiators of inflammation: A unifying explanation for diverse array of environmental stimuli to produce cutaneous inflammation. Lancet 1991;337:211-214.

Barker J, Jones ML, Swenson CL, Sarma V, Mitra RS, Ward PA, Fantone JC, Nickoloff BJ: Regulation of keratinocyte-derived monocyte chemotaxis and activating factor (MCAF) by interferon-gamma. J Immunol 1991;146:1192-1197.

Barker J, Jones ML, Mitra RS, Fantone JC, Dixit VM, Nickoloff BJ: Modulation of keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. Am J Pathol 1991;139:869-876.

Stoff T, Mitra R, Sarma V, Dixit V, Nickoloff B: Keratinocyte activation following T-lymphocyte binding. J Invest Dermatol 1992;98:92-95.

Nickoloff BJ, Mitra RS, Green J, Thompson C, Turka L, Chime Y, Shimizu Y: Discordant expression of CD-28 ligands-BB-1 and B7 on cultured keratinocytes and psoriatic cells in-vivo. Am J Pathol 1993;142:1029-1040.

Nickoloff BJ, Mitra RS, Shimizu Y, Thompson C, Turka LA: Accessory cell function of keratinocytes for superantigens: Dependency on LFA-1/ICAM-1 interaction. J Immunol 1993;150:2148-2159.

Nestle FO, Zheng X-G, Thompson CB, Turka LA, Nickoloff BJ: Characterization of Dermal Dendritic Cells Obtained from Normal Human Skin Reveals Phenotypic and Functionally Distinctive Subsets. J. Immunol (In press), 1993.

A three month sabbatical leave will be taken in June-August, 1994 (off site assignment).
Proportion of time to be devoted to this research and to other research: 80%
Identification of current or prior Federal Government service: None.
Number of graduate students: None.

BIOGRAPHICAL SKETCH

NAME: James M. Wilson, M.D., Ph.D. POSITION TITLE: Director, Institute for Human Gene Therapy

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Albion College, Albion, MI	B.A.	1977	Chemistry
University of Michigan, Ann Arbor, MI	Ph.D.	1984	Bio chem
University of Michigan Medical School	M.D.	1984	Medicine

Professional Experience:

1984-86	Residency, Massachusetts General Hospital, Medical Services
1986-88	Postdoctoral Fellowship, Internal Medicine and Biological Chemistry, University of Michigan
1988-91	Assistant Professor, Internal Medicine and Biological Chemistry, University of Michigan
1991-93	Associate Professor, Internal Medicine and Biological Chemistry, University of Michigan
1993-	Professor and Chairman of Molecular and Cellular Engineering, University of Pennsylvania
1993-	Professor, The Wistar Institute
1993-	Director, Institute for Human Gene Therapy, University of Pennsylvania

Publications:

Wilson JM, Johnston DE, Jefferson DM, and Mulligan RC. Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA. 85:4421-4425, 1988.

Wilson JM, Birinyi, Salomon RN, Libby P, Callow AD, Mulligan RC: Implantation of vascular grafts lined with genetically modified endothelial cells. Science, 1344-1346, 1989.

Wilson JM, Danos O, Grossman M, Raulet DH, Mulligan RC: Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells. Proc Natl Acad Sci. 87:439-443, 1990.

Krause JC, Mayo-Bond L, Rogers CE, Weber KL, Todd RF III, Wilson JM. An in vivo animal model of gene therapy for leukocyte adhesion deficiency. J Clin Invest. 88:1412-1417, 1991.

Chowdhury JR, Grossman M, Gupta SJ, Chowdhury NR, Baker JR, and Wilson JM: Long term improvement of hypercholesterolemia after ex vivo gene therapy in LDL receptor deficient rabbits. Science. 254:1802-1805, 1991.

Yang Y, Raper SE, Cohn JA, Engelhardt JF, and Wilson JM. An approach for treatment hepatobiliary disease of cystic fibrosis by somatic gene transfer. Proc Natl Acad USA. 90:4601-4605, 1993.

Roessler BJ, Allen ED, Wilson JM, Hartman JW and Davidson BL: Adenoviral mediated gene transfer to rabbit synovium in vivo. J Clin Invest. 92:1085-1092, 1993.

Kozarsky K, Grossman M and Wilson JM: Adenovirus mediated correction of the genetic defect in hepatocytes from patients with familial hypercholesterolemia. Sorn Cell and Mol Gen, in press, 1993.

Engelhardt JF, Simon RH, Yang Y, Zepeda M, Pendleton SW, Doranz B, Grossman M and Wilson JM: Adenovirus mediated transfer of the CFTR gene to lung of non-hjuman primates: Biological Efficacy study. Human Gene Therapy, in press, 1993.

No sabbaticals or extended leaves are anticipated.

Proportion of time to be devoted to this research and to other research: 95%

Identification of current or prior Federal Government Service:

External Strategic Planning Task Force, Member Molecular Medicine Panel, June 23-25, 1992.
NHLBI Strategic Planning Task Force on Gene Therapy, Chairman, June 30, 1992
NIH GCRC Evaluation Panel, August 17-18, 1993.
NIH NCRR Strategic Plan Workshop, September 28-30, 1993.

Number of graduate students: Four

BIOGRAPHICAL SKETCH

NAME: Stephen L. Eck POSITION TITLE: Instructor

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Kalamazoo College, Kalamazoo, MI	B.A.	1975	Chemistry
Harvard University, Cambridge, MA	M.S., PhD	1981	Chemistry
University of Mississippi, Jackson, MS	M.D.	1987	Medicine
University of MI Hosp, Ann Arbor, MI	Res/Fel	1992	Int Med/Hem Onc
University of MI Hosp, Ann Arbor, MI	Lecturer	1993	Int Med/Hem Onc
University of PA, Med Center, Philad, PA	Instructor	Present	Medicine-Hem Onc

Professional Experience:

1975-76 Teaching Assistant in Chemistry, Harvard University, Cambridge, MA
1976-77 Physical Science Instructor, Southern West Virginia Community College
1977-78 Senior Teaching Fellow in Chemistry, Harvard University, Cambridge, MA
1981-83 Senior Research Scientist, Monsanto Company, St. Louis, MO
1983-87 Research Associate, Dept of Biochemistry, University of Mississippi
1987-89 Intern and Resident, Internal Medicine, University of Michigan Hospitals
1989-92 Hematology/Oncology Fellow, University of Michigan Hosp, Ann Arbor, MI
1992-93 Lecturer, Department of Internal Medicine, University of Michigan
1993- Instructor, Department of Internal Medicine, University of Pennsylvania

Publications:

Wender PA, and Eck SL (1977). Organobiscuprates. A single-step spiroannelation method. Tetrahedron Letters, 18 (14) 1245-1248.

Wender PA, and Eck SL (1982). The Olefin Metathesis/Transannular Ene Sequence: A Method for the Stereo-controlled Synthesis of Trans-Decalin Derivatives. Total Synthesis of Warburganal. Tetrahedron Letters, 23(18) 1871-1874.

Wilson VE, Eck SL, and Bates ER (1991). Diagnosis and Management of Acute Myocardial Infarction Complicating Systemic Lupus Erythematosis. Chest, 101:420-424.

Eck SL and Nabel GJ (1992). Antisense Oligonucleotides for Therapeutic Intervention. Current Opinion in Biotechnology, 2:897-904.

Eck SL, Morse JM, Janssen DA, Emerson SG and Markovitz DM (1993) Angioedema Presenting as Gastrointestinal Symptoms. Am J Gastro, 88:436-439.

Eck SL, Perkins ND, Carr DP and Nabel GJ (1993). The Inhibition of Phorbol Est Induced Cellular Adhesion by Competitive Binding of NF-kB In Vivo. Mole. Cell Biol., 13:6530-6536.

No extended periods of leave such as sabbatical are planned during the time frame of this grant.
Proportion of time to be devoted to this research and to other research: 90%
Identification of current or prior Federal Government Service: None
Number of graduate students: None

Assistants

There will be four assistants involved in the proposed project. A Research Assistant II with a scientific background of a college degree plus 2 years experience working with tissue culture and cellular immunology to perform experiments with Drs. Turka and Chang. A Radiation Oncology Technician with a scientific background of a college degree plus 2 years experience working with tissue culture will perform experiments with Dr. Ethier. A Research Nurse with a bachelor of science in nursing and clinical research experience will work with Dr. Smith on the clinical trial. A Data Manager with experience in clinical trials monitoring will work with Dr. Smith on the clinical trial.

Other Personnel

Amy Laura, Research Secretary III will provide secretarial support. Julie Goldstein, Grant Manager will administer the grant.

Name: John W. Smith II, M.D.

Name: Stephen P. Ethier, Ph.D. Active: X
a. NIH CA40064-07 PI: Stephen P. Ethier, Ph.D.
Title: Growth factor independence in mammary neoplasia
b. Principal investigator % effort: 50%
c. 04/01/92 - 03/30/94 $538,442
d. 04/01/92-03/30/94 $138,417
e. The grant examines the cellular and molecular mechanisms of growth factor dependence in rat mammary carcinoma cells.
f. None
g. No adjustments will be necessary.

Name: Stephen P. Ethier, Ph.D. Active: X
a. NIH R01-CA57601-01 PI: Stephen P. Ethier, Ph.D.
Title: B7 gene transfer for breast cancer immunotherpy
b. Co-investigator % effort: 25%
c. 07/01/93 - 06/30/97 $650,550 (total)
d. 07/01/93 - 06/30/94 $142,847 (ttoal)
e. to clone rat B7 gene and prepare B7 expression vectors for transfection into rat mammary carcinoma cells; to transfect rat mammary carcinoma cells with B7 to determine its ability to induce tumor cell immunogenecity; to transfect human breast cancer cells with human B7 expression vectors to improve human breast cancer cell culture methods to allow B7 gene transfer into cells from a large portion of breast cancer specimens.
f. None
g. No adjustments will be necessary.

Name: Stephen P. Ethier, Ph.D. Active: X
a. NIH P30 CA4592 PI: Max Wicha, M.D.
Title: Breast Care Core
b. Co-Investigator % effort: 5%
c. 06/01/92 - 05/31/94 $50,000
d. 06/01/92 - 05/31/94 $50,000
e. to use cell culture methods and polymerase chain reaction based methods for detection of occult metastatic breast cancer cells in bone marrow aspirates of advanced breast cancer patients.
f. None
g. No adjustments will be necessary.

Name: Laurence A. Turka Active: X

a. NIH:1R01 DK44737-01 P.I. Laurence A. Turka
Title: Accessory Molecules in Thymic Maturation
b. Principal Investigator % effort: 50%
c. 92/07/01 - 95/06/30 $438,207
d. 93/07/01 - 94/06/30 $165,323
e. Does signal transduction initiated by CD4 or CD8 enhance the process of TCR-mediated cell death in immature "double positive" thymocytes;2) Does CD28 stimulation deliver a second signal which rescues thymocytes from programmed cell death? 3) Does blocking the CD28 pathway during thymic ontogeny prevent the appearance of mature thymocytes.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka Active: X
a. University of Michigan Multipurpose Arthritis Center PI: Gabriel Nunez
Title: Function and Regulation of BCL-2 proto-oncogen in T cell Development
b. Co-investigator % effort: 5%
c. 93/01/01 - 95/12/30 $149,785
d. 93/01/01 - 93/12/30 $49, 242
e. 1) Characterization of signals that regulate bcl-2 expression in developing thymocytes. 2) Examine the function and regulation of bcl-2 in models of programmed T cell death and toleration.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka Active: x
a. NIH, PSA: 1K11AI01052-01 PI: Jeanne Gratiot-Deans
Title: Regulation of Programmed Cell Death in Thymic Selection
b. Sponsor % effort: 0%
c. 92/08/01 - 97/07/31 $407,750
d. 93/08/01 - 94/07/31 $84,000
e. 1) Determine whether stimulation of immature thymocytes via the CD4 or CD8 molecules enhances the process of TCR-mediated cell death? 2) Does CD28 stimulation deliver a second signal which rescues thymocytes from programmed cell death? 3) What are the differences in protein expression and/or protein phonsphorylation characteristic of cells induced to proliferate vs. cells induced to undergo DNA apoptosis.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka Active: X
a. NIH, NIAMS SCOR in RA PI: Joseph Holoshitz
Title: The CD28 Activation Pathway and Superantigens in Rheumatoid Arthritis
b. PI of subproject 5 % effort: 15%
c. 92/09/01 - 97/08/31 $294,837
d. 93/09/01 - 94/08/31 $54,435
e. 1) Examine the ability of the CD28 pathway of T cell activation to provide a costimulatory signal for the response to superantigens. 2) Determine if blockade of the CD28 pathway will suppress the T cell response to superantigens. 3) Examine whether in vivo inhibitions of CD28 activation can prevent the development of collagen-induced arthritis in mice. 4) Determine whether blockade of the CD28 pathway can ameliorate an established arthritic process.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka — Active: X
a. National Cancer Institute/1R01CA61225 — PI: Laurence A. Turka
Title: B7 Gene Transfer for Breast Cancer Immunotherapy
b. Principal Investigator — % effort: 15%
c. 93/07/01 - 97/06/30 $535,094
d. 93/07/01 - 94/30/06 $180,000
e. 1) To complete initial experiments and clone a full length rat B7 cDNA: 2) determine effect of B7 expression on a rat mammary carcinoma nodel. 3) examine in vitro immune respones induced by B7-expressing human breast cancer; and 4) refine methods to establish human breast cancer cell lines.
f. Budgetary overalp exists for Dr. Judge's salary. If both grants are funded this money will be used for another post-doctoral fellow.
g. No adjustments will be necessary.

Name: Laurence A. Turka, M.D. — Active: X
a. Bristol-Myers Squibb — PI Laurence A. Turka
Title: Prevention of Allograft Rejection with CTLA4Ig
b. Principal Invstigator — % effort: 0%
c. 8/24/93 - 8/23/94 $78,952
d. Same
e. 1) to determine whether immunosuppression induced by the protocol which leads to long-term allograft survival, DST on day 0 followed by CTLA4Ig on day 2, is antigen specific. 2) To further study the use of CTLA4Ig in combination with cyclosporine.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka, M.D. — Pending: X
a. National Cancer Institute — PI: Samir M. Hanash
Title: Phosphoprotein alterations in malignancy
b. Co-investigator — % effort: 15%
c. 92/12/01 - 97/11/30 $1,494,502
d. 92/12/01 - 93/12/31 $258,126
e. 1) to determine the mechanism of phyophorylation/dephosphorylation of Op18 in normal and in leukemic cells. 2) to determine the effect in mice of targeted deletion by homologous recombination of the Op18 gene for which we have previously determined the complete human genomic sequence.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka, M.D. — Pending: X
a. NIH — PI: Tom Judge, M.D.
Title: B7 gene transfer for breast cancer immunotherapy
b. Sponsor — % effort: 0%
c. 93/01/10 - 95/06/30 $61,775
d.
e. 1) complete initial experiments and clone a full length rat B7 cDNA. 2) establishment of B7 expressing cell lines. 3) in vivo transplantation studies of B7 expressing cell lines.
f. No significant overlap.
g. No adjustments will be necessary.

Name: Laurence A. Turka, M.D. — Pending: X
a. National Kidney Foundation — PI: Laurence A. Turka
Title: Inhibition of Allograft Rejection with CTLA4Ig
b. Principal Investigator — % effort: 0%
c. 07/01/94 - 06/30/97 $150,000
d.
e. 1) examine the cytokine profiles present in animals treated with CTLA4Ig. 2) determine whether the establishment of a recipient-donor chimeric state might play a role in engraftment in this model. 3) examine other operative mechanisms such as the development of suppressor cells, anergy or GVH effects.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Pending: X
a. NIH P50 SPORE
PI: Richard Boland, MD
Title: Spore in gastrointestinal cancer
b. Co-Principal investigator
% effort: 10%
c. 93/10/01 - 96/09/30 $2,192,772
d. 93/10/01 - 94/09/30 $733,661
e. Clinical trial on adoptive immunotherapy with in vivo primed, in vitro anti-CD3/IL-2 activated T cell for colon cancer.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. NCI: T32 CA09672-01
PI: Alfred E. Chang, MD
Title: Surgical Oncology Training in Tumor Immunology
b. Program Director
% effort: 5%
c. 91/07/01 - 96/06/30 $652,212
d. 93/01/01 - 93/12/31 $105,057
e. Basic research fellowsh for surgical oncologists in training.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. NCI: 2P30 CA46592-04
PI: Max Wicha, MD
Title: University of Michigan Cancer Center
b. Program Director, GI Oncology Program
% effort: 10%
c. 91/07/01 - 96/06/30 $4,705,953
d. 93/01/01 to 93/12/31 $2,070,325
e. This is a Cancer Center grant, mostly for administrative work.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. American Cancer Society Inc.
PI Alfred E. Chang, M.D.
Title: Activation and Propagation of T cells for Cancer Therapy
b. Faculty Research Awardee
% effort: N/A
c. 92/07/01 to 97/06/30 $205,000
d. 93/01/01 to 93/12/31 $37,000
e. Faculty salary support for basic and clinical research in adoptive immunotherapy.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. NIH PO1 CA59327-01
PI: Alfred E. Chang
Title: Genetic Approaches to Cancer Immunotherapy
b. Program Director
% effort: 20%
c. 92/09/30 to 96/09/30 $2,517,743
d. 93/09/30 to 94/09/30 $599,695
e. Experimental and clinical models of genetic modulation of immunotherapy.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. NCI: 1R01 CA57815-01
PI Alfred E. Chang
Title: Immune responses to genetically modified tumors
b. Principal investigator
% effort: 10%
c. 92/09/10 to 94/08/31 $279,097
d. 93/09/10 to 94/08/31 $147,109
e. This project investigates the functional antigenicity of gene modified tumors in stimulating effector T cells for adoptive immunotherapy.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Pending: X
a. NIH R01
PI: Suyu Shu, Ph.D.
Title: Superantigen activated T cells in tumor immunotherapy
b. Co-investigator
% effort: 5%
c. 94/04/01 to 99/03/31 $1,183,006
d. 94/04/01 to 95/03/31 $222,674
e. 1) identify TCR VB gene usage in the pre-effector cell response through activation with superantigens. 2) to analyze immunologic mechanisms involved in superantigen induced differentiation of pre-effector cells and tumor eradication mediated by activated cells. 3) to evaluate the vivo effects of superantigens on the development of antitumor immunity and on tumor-induced specific suppression. 4) to investigate the potential benefits that may be derived from superantigen based gene therapy.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

Name: Alfred E. Chang
Active: X
a. NCI: R01 CA58927-01
PI: Suyu Shu
Title: Activation of T cells for tumor specific immunotherapy
b. Co-investigator
% effort: 10%
c. 92/12/01 to 97/11/30 $1,248,393
d. 93/01/01 to 93/12/31 $239,982
e. Experimental models of T cell activation and mechanisms of T cell mediated antitumor effects.
f. No scientific or budgetary overlap.
g. No adjustments will be necessary.

UNIVERSITY OF
PENNSYLVANIA
MEDICAL CENTER

**Institute for
Human Gene Therapy**

University of Pennsylvania School of Medicine
Hospital of the University of Pennsylvania

November 23, 1993

Stephen Ethier, Ph.D.
Department of Radiation Oncology
University of Michigan Medical Center
1331 Ann Street
Ann Arbor, MI 48109

Larry Turka, MD.
Department of Medicine
Division of Nephrology
MSRB II, Room
1150 W. Medical Center Drive
Ann Arbor, MI 48109

John Smith, M.D.
Department of Internal Medicine
Division of Hematology/Oncology
3118 Taubman Center
1500 E. Medical Center Drive
Ann Arbor, MI 48109

Dear Drs. Ethier, Turka, and Smith:

Pursuant to our recent discussions concerning the development of a clinical Phase I trial of human B7 expressed in breast cancer cells, we pleased to supply you with recombinant adenovirus (Ad 5 serotype) expressing human B7. The preparation of this virus is described in the accompanying documents. In brief, replication defective adenoviral vectors expressing human B7 (hB7) will be prepared as follows. We cloned the hB7 cDNA by RT-PCR using mRNA from Daudi cells and primers designed to amplify the entire protein-coding domain based on the published sequence. After cloning, the cDNA was sequenced to insure that it encoded normal human B7 and that no mutations had been introduced during PCR. The hB7 cDNA was excised from the plasmid as blunt-ended fragments and ligated into the pAdCMV-*lacZ* plasmid after removing the *lac Z* sequence with Not I and blunting the plasmid ends with the Klenow fragment of DNA polymerase I. After determining subclones with the correct orientation of the B7 cDNA by restriction digestion, we obtained pAdCMVhB7, that will be used to prepare recombinant adenoviruses.

Wistar Institute, Room 204 • 36th and Spruce St. • Philadelphia, PA 19104-4268 • 215-898-3000 • FAX 215-898-6588

University of Pennsylvania Health System

The adenovirus vector will be generated by homologous recombination between pAdCMVhB7 and sub360 adenoviral DNA (E1a$^-$ and E3$^-$,) in 293 cells (E1a$^+$). Recombination between linearized pAdCMVhB7 and sub360 adenoviral DNAs will generate a defective adenovirus genome in which the B7 cDNA, under the control of the CMV immediate-early promoter, replaces the adenovirus E1a gene. This defect will be complemented in *trans* in cells that express a transfected E1a gene (e.g. 293 cells) to generate replication-defective adenovirus virions. Specifically, NheI-linearized pAdCMVhB7 plasmid DNA and XbaI/ClaI-digested sub360 DNA will be co-precipitated with calcium phosphate and used to transfect 293 cells in serum-free media. Non-linearized sub360 DNA will be transfected as a positive control for transfection and adenovirus generation, and linearized sub360 DNA will be transfected alone (without B7 plasmid DNA) as a background control for transfection and adenovirus generation in the absence of recombination. The following day, the cells are overlaid with 1.6% agar in media. The overlay is repeated on day 5, and on day 9 plaques (focal areas of low cell density and lysed cells, indicative of spreading adenovirus infection) are visualized by neutral red staining. Individual plaques are harvested and used to infect fresh plates of 293 cells. After two rounds of plaque purification, the virus will be quantitatively expanded on multiple plates of 293 cells and purified by CsCl gradient centrifugation. The ability of the recombinant adenovirus to express B7 will be assayed by testing their ability to confer B7 surface expression on Hela cells (normally B7-) after a two hour exposure to virus and overnight incubation. This work will be completed in the Human Applications Laboratory of the Institute for Human Gene Therapy, where adenovirus preparations are routinely performed under condition of "good laboratory practice" and "good manufacturing practice". Appropriate tests will be performed to ensure that the virus prepared for human use will meet the criteria for FDA certification. (See accompanying documents for details). This procedure has previously been carrier out for another recombinant adenovirus which is currently in clinical trials. The costs of virus preparation and certification are detailed in the accompanying materials.

We look forward to working with you in this worthwhile endeavor.

Sincerely,

James M. Wilson, M.D., Ph.D.
Director

Stephen L. Eck, M.D., Ph.D.
Instructor, Department of
Hematology/Oncology

SLE:dlj

The University of Michigan
Medical School

DEPARTMENT OF PATHOLOGY

Brian J. Nickoloff, M.D., Ph.D.
Associate Professor of Pathology & Dermatology

November 15, 1993

John Smith, M.D.
Associate Professor of Medicine
University of Michigan Medical School
Ann Arbor, MI

Dear John,

I would be delighted to collaborate with you in examination of lymph node and skin biopsies of patients treated with your protocol of B7-transfected breast cancer cells. My laboratory has extensive experience in histological and immunohistochemical evaluation of skin biopsies, including the use of 2-color immunohistochemistry. A co-investigator of this grant, Larry Turka, already has a close working relationship, and this should facilitate my involvement in this exciting project.

We look forward to hearing from you regarding this work.

Best regards,

Brian J. Nickoloff, M.D., Ph.D.
Associate Professor of Pathology
and Dermatology
University of Michigan Medical School

Enclosures:

L111593a.BJN/csm

1301 Catherine Rd. • Room M-5214 Medical Science I • Ann Arbor, MI 48109-0602 • (313)764-3190 • FAX:(313)764-2397

The University of Michigan
Medical Center
Ann Arbor, Michigan 48109-0680

Department of Internal Medicine
Division of Hematology and Oncology
5510 MSRB I
1150 W. Medical Center Dr.
(313) 747-3421

November 24, 1993

John W. Smith II, M.D.
Associate Professor
Department of Internal Medicine
Division of Hematology/Oncology
3119 Taubman Center
1500 E. Medical Center Drive
Ann Arbor, MI 48109-0374

Dear John:

This letter officially confirms my intention to collaborate with you on the B7 transfected autologous breast cancer vaccine trial. As Director of the Breast Oncology Program at the University of Michigan, I will be able to identify potential patients for enrollment onto this trial and will encourage the clinicians involved in the Program to consider this study as an option for appropriate patients.

I look forward to participating in this exciting clinical trial.

Sincerely,

Barbara L. Weber, M.D.
Assistant Professor, Internal Medicine
Director, Breast Oncology Program

Facilities and Equipment

A. University of Michigan Medical Center:

The University of Michigan Medical Center includes several hospitals totaling more than 1000 beds, out-patient facilities, Schools of Medicine, Nursing, and Public Health, the Howard Hughes Institute for Molecular Biology, and extensive and diverse research facilities. The Medical Center, located on the campus of the University of Michigan in Ann Arbor, serves as a tertiary referral center for patients from much of Michigan, northern Ohio, Indiana, western Ontario, and parts of Illinois. With the completion of the new $285 million Adult General Hospital and the completion of the remodeling of Mott Children's Hospital and Women's Hospital, the Medical Center is among the best equipped University Hospital complexes in the world.

B. Laboratories and Major Equipment:

1. Dr. Chang's laboratory space consists of 2000 square feet in the Medical Science Research Building I (MSRB I), Rooms 1518, 1522, and 1526 on the University of Michigan campus, used jointly by Drs. Chang and Shu will be the site for the proposed study. The 500 square feet laboratory space in Room 1518 is used exclusively for activation of human lymphocytes for clinical therapy. In Dr. Chang's laboratory (MSRB I, 1518) are two 6-foot laminar airflow hoods, two Sorvall centrifuges (one table-top and one floor model), three CO_2 incubators, and one freezer. In Dr. Shu's laboratory next door (MSRB I, 1526) are a Revco freezer and liquid nitrogen freezer. A gamma counter and a multiple harvester are also available.

2. Dr. Ethier's laboratory is in the Cancer Biology Division of the Department of Radiation Oncology. Dr. Ethier's laboratory is approximately 1200 square feet that consists of a main laboratory and a separate tissue culture laboratory. This lab is fully equipped for the preparation of tissues and human breast cancer cell isolation. The laboratory has 2 laminar flow hoods, 4 water-jacketed CO_2 incubators, numerous water baths including 2 shaking water baths that are used for enzymatic dissociation of breast tissues, an inverted phase contrast microscope, bench top refrigerated centrifuges and microfuges. At the present time, human breast cancer cells maintained frozen in our cell bank are stored in the Cancer Biology Divisions liquid nitrogen freezers.

3. Dr. Turka's laboratory (1560 MSRBII) consists of 1000 square feet of space fully equipped for cell culture, bacterial culture and plasmid preparation, agarose and polyacrylamide gel electrophoresis, Northern, Southern and Western blotting, and immunofluorescent staining. Flow cytometric analyses will be performed on a FACScan located within the adjacent building.

C. Clinical:

1. Clinical Research Center: Within the new Adult General Hospital is a 16-bed Clinical Research Center funded by the NIH (Grant 115MO1-RR00042). This is the site where all in-patient diagnostic and therapeutic clinical studies will be performed in this project. This center has nurses and paramedical personnel experienced, and this facility is an excellent location for the conduct of therapeutic clinical studies. This unit will provide basic hospitalization without additional expense for patients who are on the research protocol which has been approved by the CRC Review Committee for scientific value and safety. In addition, the CRC, in cooperation with the Hospital, will pay for a variety of routine diagnostic tests as well as those involving high technology. In addition, the CRC will pay for selected out-patient studies as well. The availability of the CRC substantially reduces the cost of the conduct of clinical trials such as those proposed. Furthermore, since the proposed studies have been approved by the CRC, they are eligible for biostatistical consultation without charge. We have incorporated this into our budget to allow for further cost savings.

The Human Applications Laboratory is an FDA approved BL2 facility with many features of a BL3. The laboratory environment is controlled by a Liebert Challenger 3 HEPA air filtration system which maintains appropriate pressure differentials and directional air flow. Access to the laboratory is strictly controlled. There are three Baker Class II Type A/B3 Laminar Flow Tissue Culture Hoods. There are six Forma Scientific CO2 incubators which have a Gas Guard monitoring system. Decontamination/Sterilization needs are met by a Program Control BIOFOE Sterilizer, by Getinge, that is within the lab. Purified water is supplied by an in lab Milli-Q UF system. There is a Nikon Phase microscope. Refrigeration storage facilities consists of two liquid nitrogen freezers, two -70°C freezers, one -20°C freezer and one 4°C refrigerator. Centrifugation needs are met by a Beckman Spinchron (refrigerated), and IEC centrifuge, and two Eppendorf (refrigerated) microfuges. Four temperature controlled water baths are also available. GMP and GLP practices are followed at all times.

2. Breast Care Center: The Breast Care Center (BCC) of the University of Michigan Cancer Center, under the direction of Dr. Barbara Weber, will be the focal point of this investigation. This multidisciplinary diagnostic, therapeutic, and clinical research unit, involving more than 40 participants, was established in 1985 to promote interdisciplinary care and research for breast disease patients. Over 3200 BCC patient visits are anticipated for the year 1993. All breast cancer patients cared for at the University of Michigan are initially seen in the BCC. All new cancer cases seen are discussed at a weekly multidisciplinary conference. Studies of the type proposed have been performed previously under the auspices of the BCC. Patients are referred to the BCC by practitioners at the University of Michigan, the University of Michigan Turner Geriatric Center, the University of Michigan Breast Cancer Detection Center (BCDC), community based practitioners throughout a three state region, and patient initiative. To improve the access of low income and minority women to the clinical services, facilities, resources, and research efforts of the BCC, the BCC is participating with the Washtenaw County Public Health Department in a federally sponsored program (Title XV) aimed in part at improving breast cancer screening access and compliance in underprivileged women. Up to 500 women yearly will be screened in the BCC under this program, and definitive diagnostic and therapeutic care will be available to those in whom conditions requiring intervention are identified. The Turner Geriatric Center is a University of Michigan dedicated geriatric facility that follows approximately 1200 women yearly and that has a strong commitment to breast cancer screening in the elderly. Turner Clinic patients newly diagnosed with breast problems are encouraged to seek their definitive care through the BCC. The BCDC is a mammographic and physical examination breast cancer screening program under the auspices of the University of Michigan that performs in excess of 12,000 screening procedures annually.

   Between its inception February 1, 1985 and December 31, 1992, there were over 17,650 patient visits to the BCC, and the Center continues to grow at an annual rate of approximately 5%; more than 3200 visits are anticipated for the year 1993. The BCC weekly patient care conference will act as a protocol clearinghouse. It will permit regular assessment of patient eligibility and enrollment, ongoing clinical monitoring, and facilitation of data collection in a comprehensive and standardized fashion. The BCC database will be available to assist with patient monitoring, follow-up care, and clinical data acquisition.

D. University of Michigan Comprehensive Cancer Center:

1. Cancer Center: The University of Michigan Cancer Center is one of 27 designated Comprehensive Cancer Centers. This Center, directed by Dr. Max Wicha, was established in 1987 and is comprised of 275 members supported by over $50 million annually in research grant support. The Multidisciplinary Breast Cancer Program, headed by Dr. Barbara Weber, is a prominent program of the Cancer Center. A new $88 million program facility which will house the Cancer Center Multidisciplinary Clinics and laboratories is currently under construction. Completion is expected in 1996.

The Cancer Center is an important resource and provides a rich environment for the conduct of the proposed clinical studies. The Clinical Trials Office (CTO) of the University of Michigan Cancer Center, has 3 major sections: a) protocol/data management development, b) Cancer Nursing, c) Experimental Pharmacy. The CTO will serve key protocol/data management and experimental pharmacy functions for the execution of the clinical aspects of this proposal. The Protocol/Data Management Section provides a centralized administrative and organizational structure with expertise in both data and protocol management. It assists investigators in protocol and consent preparations for IRB submission and ensures compliance with all IRB and humans subjects protections. The Cancer Nursing Section provides coordination of patient management for those patients entered on Cancer Center approved institutional clinical research protocols. The Experimental Pharmacy a centralized pharmacy-based investigational drug control service to comply rigidly with FDA and NCI drug accountability guidelines for oncologic agents as well as other investigational drugs. Through the Clinical Trials Office, all aspects of clinical protocol development and execution are monitored and standards of excellence maintained. Data manager staffing for this study is provided by the Protocol Office of the Division of Hematology/Oncology, which works cooperatively with the CTO.

E. Office: Dr. Smith occupies a 100 square feet office in the Taubman Center and is equipped with a Macintosh Centris 650, which will be used for maintaining files and records related to this study.

CERTIFICATE OF ENVIRONMENTAL AND SAFETY COMPLIANCE

The offeror currently ☒ IS ☐ IS NOT in compliance with applicable national, state, and local environmental and safety laws and regulations.
(If not in compliance, attach details and evidence of approved mitigation measures.)

The offeror has examined the activities encompassed within the proposed action entitled " Gene THerapy of Human Breast Cancer "
(enter title and/or Solicitation number and Principal Investigator's name), for compliance with environmental and safety laws and regulations. The offeror states that the conduct of the proposed action ☐ WILL ☐ WILL NOT violate any applicable national, state, or local environmental or safety law or regulation.
(If a violation will result, attach details describing the nature of the violation and evidence of approved mitigation measures.)

The offeror agrees that if the work required under the proposed action at any time results in a violation of any applicable environmental or safety law or regulation, the offeror will immediately take appropriate action, to include notifying the Contracting Officer, and coordinating with the appropriate regulatory agencies.

Kenneth C. Schatzle
(Name of Official Responsible for Environmental and Safety Compliance)

Signature

Director EH&S
(Title)

11/23/93
Date

University of Michigan
Occupational Safety & Environmental Health
(Name of Organization)

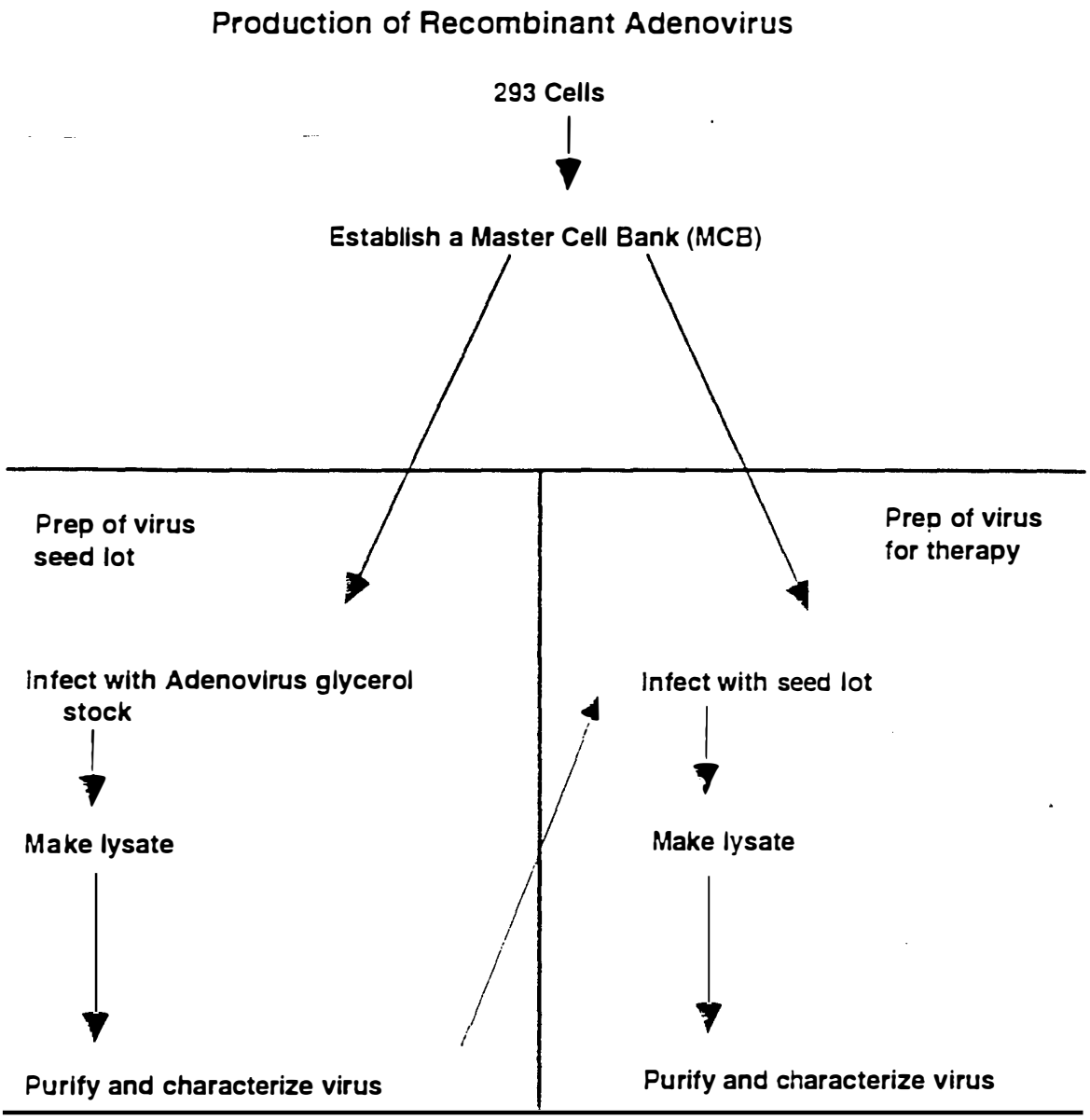
IV. Generation and production of certified recombinant adenovirus

A. Overall Strategy

The overall strategy for the production of recombinant adenovirus for gene therapy clinical trials is summarized in a schematic below. A more detailed description of the vector construction can be found in the vector core section. An overall strategy for the production and validation of recombinant adenoviruses is presented in Figure 3.

Similar to the generation of recombinant retroviral gene therapy reagents, adenoviral reagents have been constructed to provide "cassette" oriented approaches to their production. A recombinant adenoviral vector was constructed from a modified adenovirus type 5 in which the minigene of interest is inserted in place of E1a and E1b. This vector is cotransfected along with the large fragment of the enzyme-restricted Ad5 DNA into 293 cells. 293 cells are a human kidney cell line that contains a functional E1a gene and provides a trans-acting E1a protein in order to allow for homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infectious virions and formation of plaques. A Master Cell Bank (MCB) of 293 cells have been previously established and evaluated for performance, in terms of production of recombinant adenoviruses, and for the absence of other pathogenic contaminants (see section IV.B.1). Individual plaques are isolated and amplified in the 293 cells, viral DNA isolated, and recombinant adenoviral plaques containing the functional DNA would be identified by restriction cleavage and Southern blot analysis. The MCB is then infected with the crude viral lysates and plaque-purified a second time in order to generate a seed lot lysate. Virus is then purified and cryopreserved in aliquots. Titers of viral stocks are determined by a plaque assay using 293 cells. The purified seed lot lysate is subjected to safety testing as described in section IV.B.2. The MCB is then plated again and infected with the certified viral seed lot. Lysates are harvested from the infected cells and virus is purified from the lysate, cryopreserved, and lots will be evaluated as described below in section IV.B.3. The purified adenoviral DNA is then subjected to sequencing as described in section IV.B.4.

Figure 3



B. Quality Assurance and Quality Control

A three-stage test program has been designed to assess the cell bank, product intermediate (cell lysate) and the purified product (virus). The quality assurance and quality control of each stage is documented by the tests listed below. The standard operating procedures for each step process follows the list of test descriptions.

Process	Testing
293 Master Cell Bank	Characterization of the cell bank

Infect 293 cells with adenovirus	Characterization of intermediate cell lysate
Seed lot	Adenovirus seed lot virus testing (adventitious contaminants)
Purified Product	Product Testing

1. Certification of #293 Master Cell Bank

The following tests have been performed on aliquots of the 250 ampules of Master Cell Bank that had been established for the cell line #293. All tests have been negative except for the tumorigenicity assay which scored positive as expected.

Establishment and Preservation of a Cell Bank. The cell line, #293, was cultured to provide a sufficient quantity of cells for the preparation of a cell bank. Prior to cryopreservation of the cells, samples were prepared to test for the presence of bacterial and fungal contaminants, sterility test and to test for the presence of agar cultivable and non-cultivable mycoplasmas. These assays were performed to assure the absence of microorganisms in the cell line prior to establishment of the cell bank.

Test for the Presence of Agar-Cultivable and Non-Cultivable Mycoplasmas. The purpose of this assay is to detect the presence of any agar-cultivable or non-cultivable mycoplasma species that may be present. The semi-solid broth and agar media utilized provide the necessary nutrients for the growth of these mycoplasma species. Two types of agar medium are utilized to ensure the detection of both fastidious and easily cultivated strains. Subcultures greatly increase the likelihood of detection even when low levels of mycoplasma contamination are present. In addition, non-cultivable mycoplasma are detected by the use of the Hoechst stain procedure on indicator cells (Vero) when inoculated. This procedure allows for the growth of mycoplasmas in a mycoplasma free indicator cell line which then can be stained and the mycoplasma contaminants easily observed.

Test for the Presence of Bacterial and Fungal Contaminants: Sterility Test. The purpose of the sterility test is to determine the presence of one or more species of bacterial and/or fungal contaminants. This is determined by inoculating #293 into soybean-casein digest broth, fluid thioglycollate broth, Pre-reduced Peptone Yeast Glucose medium (PYG) and sabouraud-dextrose agar. Each media is designed to detect different types of organisms. The media are incubated at different temperatures and in different atmospheric conditions and examined three times during a fourteen day test period for evidence of bacterial or fungal growth. Those inoculates which cause growth in any of the media are not sterile and are reported as contaminated.

Cell Culture Identification and Characterization. In this study, cultured cells were characterized by isoenzyme and cytogenic analysis in order to confirm the species identity. Such information can be valuable as a base line for abnormalities intrinsic to the original material, possibilities of mislabeling and/or contamination involving other cell lines or the inadvertent exposure of the cell line to chemical or physical mutagens which might induce persisting chromosomal changes.

In Vitro Assay for the Presence of Viral Contaminants. The presence of viruses contaminating a cell line may be detected through the use of a battery of indicator cells

and observations of cytopathic or other visually discernible effects such as hemadsorption or hemagglutination. These indicator cells support the growth of a large number of viruses. It was the purpose of this study to examine the cell line for viral contamination through the use of six types of indicator cells. The indicator cells include MRC-5, a diploid human lung culture; Vero, a monkey kidney line; sponsor supplied cells; MDBK, a bovine kidney cell line; HeLa, a human cervical carcinoma line; and Swiss mouse embryo fibroblasts (MEF).

In Vitro Assay for the Presence of Bovine Viruses. The presence of bovine viral diarrhea virus (BVD), infectious bovine rhinottracheitis virus (IBR), bovine adenovirus type 3 (BAV3), bovine parvovirus (BPV), or parainfluenza virus type 3 (PI3) may be detected through the use of sensitive indicator cells and observations of cytopathic or other visually discernible effects. It is the purpose of this study to examine the cell line for bovine viral contamination through the use of bovine turbinate (BT) cells.

In Vitro Assay for the Presence of Porcine Parvovirus. The presence of porcine parvovirus (PPV) may be detected through the use of sensitive indicator cells and observations of cytopathic or other visually discernible effects. It is the purpose of this study to examine the test material for PPV contamination through the use of PT-1 cells.

Test for the Presence of Inapparent Viruses. The purpose of this assay is to detect viruses which do not cause a discernible effect in cell culture systems. #293 was inoculated into adult mice, guinea pigs and suckling mice. The suckling mouse portion of this assay included a subpassage of homogenized tissue after fourteen days into a new group of suckling mice followed by an additional fourteen day observation period. All animals were observed for signs of illness and any that became sick or showed any abnormalities were examined in an attempt to establish the cause of illness or death. Embryonated hens' eggs were inoculated with #293 or a negative control article by the allantoic route followed by a subpassage of allantoic fluid via the same route. Allantoic fluid from the original and subpassage eggs were tested for hemagglutination at 4° C and room temperature using guinea pig, human and chick erythrocytes. A second group of embryonated hens' eggs were inoculated with #293 or the control article into the yolk sac, followed by a subpassage of the yolk sac material into a new set of eggs, via the yolk sac route. All embryos were examined for viability.

Detection of Hepatitis B Surface Antigen (HBsAG) in Cell Culture. The presence of Hepatitis B Surface antigen (HBsAG) is indicative of the presence of the Hepatitis B virus. In order to detect small amounts of the Hepatitis B surface antigen, a third generation enzyme immunoassay (EIA) is utilized. This assay exhibits sensitivity equivalent to radioimmunoassay systems. In this study, beads coated with mouse antibody to HBsAG are incubated with #293 and mouse monoclonal Anti-HBs peroxidase conjugate. Any HBsAG present is bound to the solid phase enzyme conjugate. O-phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead and, after incubation, a yellow-orange color develops in proportion to the amount of HBsAG which is bound to the bead.

Detection of Human Immunodeficiency Virus (HIV) Retrovirus. In order to detect small amounts of infectious human immunodeficiency virus (HIV-1), susceptible cells such as human peripheral blood lymphocytes (PBL) are inoculated with the test article. Cultures are passaged and monitored for six weeks to enhance or amplify the possibility of detecting retrovirus by antigen capture (ELISA) technique. In this study, PBL's were inoculated with #293 and analyzed for six week's by antigen capture for the presence of HIV.

Southern Blot Hybridization Assay for the detection of Human Parvovirus B19 DNA. Molecular hybridization has been used for a number of years to detect specific complementary sequences of DNA or RNA. Radiolabeled probes are used to detect DNA immobilized on a solid matrix in hybridization reactions. In this study, a Southern transfer technique for the detection of the human Parvovirus B19 DNA per cellular genome is used. The probe is the XbaI/KpnI fragment of the plasmid pNPS-1.

Assay for the Detection of Retrovirus Particles by Electron Microscopic Examination. Transmission electron microscopy (TEM) is used to evaluate a fixed pellet for the presence of viral particles. These particles are identified, if possible by their size and shape. Thin sections of the cell pellet are observed and pictures documenting the findings are provided. In this study, #293 cells were examined by TEM in order to detect viral particles.

Detection of Adeno -Associated Virus (AAV). In this study, a Southern transfer technique for the detection of the adeno-associated viral DNA per cellular genome is used. The probe is the XbaI fragment of the plasmid pJMS-2. Genomic DNA isolated from #293 cells and the control DNA are subjected to digestion with XbaI/BamHI and XbaI/EcoRI.

In Vitro Assay for the Presence of Cytomegalovirus Contamination. The presence of cytomegalovirus contamination in a cell line may be detected through the use of sensitive indicator cells and observations of cytopathic or other visually discernible effects. These MRC-5 cells support the growth of cytomegalovirus. It is the purpose of this study to examine the cell line, #293, for viral contamination through the use of MRC-5 indicator cells.

In Vitro Assay for the Presence of Adenovirus. The presence of adenovirus contamination may be detected through the use of sensitive indicator cells and observations of cytopathic or other visually discernible effects with the exception of types 40 and 41, all of the human adenoviruses replicate and produce CPE in primary human neonatal kidney cells and in continuous human cell lines of epithelial origin such as HeLa, KB, and HEp-2. #293 cells support the growth of cell human adenoviruses including types 40 and 41. It is the purpose of this study to examine the cell line for viral contamination through the use of HNK indicator cells.

Evaluation of Tumor Formation. Nude athymic mice fail to mount a cell mediated response against foreign materials and therefore will support the growth of allogeneic and heterogeneic tumor cell lines, thus permitting assessment of the capability of an inoculum to form neoplasms in vivo. The nude mice were inoculated subcutaneously with approximately 1×10^7 cells of #293 and followed clinically for 28 days and necropsied.

Epstein Barr Virus Probe Assay. The purpose of this study is to detect EBV DNA that may be present in the test article as determined by Southern hybridization using a labeled probe. DNA is extracted from the test article cell pellet and blotted onto membranes. Blotted membranes are hybridized to a labeled EBV probe and detected by autoradiography. DNA from Manalwa cells are included as a positive control.

2. Certification and Characterization of Cell Lysate - Product intermediate

Each preparation of cell lysate will be pooled and aliquots will be evaluated for the following tests. Any lysate demonstrating contamination will be discarded.

- *Mycoplasma* - see above for test description

• *Sterility* - see above for test description

3. Certification of Recombinant Adenoviral Seed Lot Lysate

• *Mycoplasma* - see above for test description
• *Sterility* - see above for test description
• *In vitro virus* - see above for test description
• *Adenovirus Testing*

This assay is designed to detect the presence of human adenovirus in the test article. Indicator cell cultures are inoculated and observed periodically for CPE, a passage is performed for enhancement.

• *Limulus Amebocyte Lysate (LAL)*

The purpose of this study is to detect and quantitatively determine the gram negative bacterial endotoxin level of a test article or extract using the Limulus Amebocyte Lysate (LAL) gel-clot method for testing.

• *Adeno-associated virus* - see above for test description
• *Electron microscopy* - see above for test description
• *Presence of functional adenovirus* - test performed by investigator
• *In vitro assay for the presence of adenovirus* - see above for test description by an outside contract laboratory. Another helper virus assay has been developed by our laboratory and is described in an SOP in section IV.C.4. This is a critically important test in the characterization of both the seed lot and production lot. These helper virus assays have been developed and validated, both of which use indicator cell lines and extended passages, to detect contamination of stocks with wild type virus.

4. Certification of Recombinant Adenovirus - Final Product

• *Sterility* - see above for test description
• *General Safety* see above for test description

A general safety test will be performed on the viral supernatant. The purpose of this test is to detect the presence of extraneous toxic contaminants. The experimental design entails inoculating guinea pigs and mice intraperitoneally with the viral supernatant from #293. The animals will be observed for overt signs of ill-health or unusual response. Their weights are measured just prior to and upon completion of the test period.

• *Limulus Amebocyte Lysate (LAL)* - see above for test description
• *In vitro assay for the presence of adenovirus* - see above for test description
• *Presence of functional adenovirus* - test performed by investigator

5. Adenoviral DNA Sequencing

Viral DNA is isolated from the purified viral preparation obtained after three rounds of plaque purification. Selected areas of the viral genome are subjected to sequence analysis by an FDA approved facility. Sequence determination is performed in compliance with FDA/GLP procedures. Two areas of the genome are subjected to complete sequence analysis including 1) the 5' end of the genome spanning the 5' ITR,

the entire minigene cassette including any enhancers and promoters, the cDNA, and a portion of E2, and 2) the region surrounding deletions in E3. Regions to be sequenced are subcloned as overlapping restriction fragments into pBluescript II (Stratagene) and pGem5Zf (Promega). Nested deletion clones are generated in both directions for each of the subclones using a modified exoIII SI nuclease procedure. These deletion clones are size selected to provide complete coverage of each strand and sequenced using the dideoxynucleotide termination procedure. Internal sequencing primers are synthesized and used to close gaps between contigs and to fill in any single-stranded regions. Sequencing has been completed for the adenovirus DNA (Ad.CB-CFTR) that will be used for the CF trials.

C. Standard Operating Procedures (SOPs).

The following SOPs have been established for production of the CFTR virus. These SOPs should be useful for the isolation of essentially any adenovirus.

1. Standard Operating Procedure (SOP) for the production of the adenoviral cell line: #293

Day 0

1. Complete media is made by adding together the following components. This is done in a laminar flow hood using sterile techniques.

1000 ml Dulbecco's Modified Eagle's Medium (high glucose) - that has been warmed from 4° C in a 37° C water bath.
100 ml bovine calf serum - that has been thawed from -20° C in a 37° C water bath.
10 ml penicillin/streptomycin - that has been thawed from -20° C in a 37° C water bath.

2. Filter the above complete media through a one liter 0.45 mm filter unit. This is done in a laminar flow hood using sterile techniques.

3. Using a 10 ml sterile pipet, remove 10 ml of the filtered complete media. Place the 10 ml into a labeled 15 ml sterile test tube. This aliquot will be tested for sterility.

4. Thaw a seed lot of 293 cells by removing eight cryovials (2 ml) from -135° C and warming in a 37° C water bath.

5. While the cryovials are thawing, remove 8 x.20 ml aliquots of the filtered complete media using a 25 ml sterile pipet and add to 8 x 15 cm sterile tissue culture plates. This is done in a laminar flow hood using sterile technique.

6. When the 293 cells in each cyrovial has thawed, remove from the 37° C water bath and spray each vial with 70 % ethanol and wipe off the excess ethanol. Take the cryovials to the laminar flow hood and remove the cap from each vial using sterile techniques. Using a 2 ml sterile pipette, transfer the 293 cells from each cryovial to the tissue culture plates containing the filtered complete media.

7. Place the above tissue culture plates in a 37° C, 5 % CO_2 incubator.

Day 1

1. Remove the tissue culture plates from the incubator and place in a laminar flow hood. Using a sterile pasteur pipet aspirate the media from each tissue culture plate. Using a 25 ml sterile pipet, add fresh filtered complete media (that has been warmed to 37° C) to each plate.

2. Place the above tissue culture plates in a 37° C, 5 % CO_2 incubator.

Day 2 (or when 293 cells are confluent)

1. Remove the tissue culture plates from the incubator and place in a laminar flow hood. Using a sterile pasteur pipet aspirate the media from each tissue culture plate.

2. Using a 10 ml sterile pipet, add 10 ml of sterile PBS^- to each tissue culture plate.

3. Using a sterile pasteur pipette, aspirate the PBS^- from each tissue culture plate.

4. Using a 2 ml sterile pipet, add 2 ml of trypsin-EDTA (that has been warmed from -20° C in a 37° C water bath) to each tissue culture plate.

5. When the 293 cells begin to release from the tissue culture plates, add 8 ml of filtered complete media (that has been warmed in a 37° C water bath) using a 10 ml sterile pipet to each plate. This will inactivate the trypsin reaction.

6. Using a 10 ml sterile pipet, transfer the 10 ml of media/trypsin from each tissue culture plates to a labeled 15 ml sterile test tube.

7. Place each 15 ml test tube in a clinical centrifuge and spin down the cell pellet using setting 4 for two minutes.

8. Remove the test tubes from the clinical centrifuge and bring to the laminar flow hood. Using a sterile pasteur pipette, aspirate the supernatant from the test tubes.

9. Using a 10 ml sterile pipet, add 10 ml of filtered complete media to each test tube and resuspend the cell pellet by gently mixing the cell suspension.

10. Using a 10 ml sterile pipet, add the 10 ml of cell suspension to 90 ml of filtered complete media (there will be five individual 90 ml filtered complete media bottles for the eight test tubes of cell suspensions). Thoroughly mix the 100 ml of cells/media by gently swirling the bottle.

11. Using a 25 ml sterile pipette, add 20 ml of cells/media to a 15 cm sterile tissue culture plate. There will be a total of 40 x 15 cm sterile tissue culture plates.

12. Place the above 40 tissue culture plates in a 37° C, 5 % CO_2 incubator.

Day 4 (or when 293 cells are confluent)

1. Remove the tissue culture plates from the incubator and place in a laminar flow hood. Using a sterile pasteur pipette, aspirate the media from each tissue culture plate.

2. Repeat steps #2 through #11 that are listed under Day 2 with the exception that there will now be 40 individual 90 ml filtered complete media bottles and 40 individual sterile 15 ml test tubes and there will also be a total of 200 x 15 cm sterile tissue culture plates.

3. Place the above 200 tissue culture plates in a 37° C, 5 % CO_2 incubator.

Day 6 (or when 293 cells are ~90% confluent).

The above 293 cells can be used for the infection with any adenoviral seed lot stock

2. *Standard Operating Procedure (SOP) for the infection of 293 cells with adenoviral glycerol stock (seed lot lysate)*

1. On day 6 post-plating of 293 cells (see SOP for production of 293 cells), make the following media and solutions:

a)DMEM supplemented with 1% Penicillin/Streptomycin (PS)
b)DMEM supplemented with 15% FBS and 1% PS
c)10 mM Tris-HCl, pH 8.1

2. Thaw a seed lot of the adenoviral glycerol stock (5×10^{11} pfu/ml) by removing a cryovial from -80° C and warming in a 37° C water bath.

3. While the cryovial is thawing, remove the tissue culture plates from the incubator and place in a laminar flow hood.

4. When the adenoviral glycerol stock in the cyrovial has thawed, remove from the 37° C water bath and spray each vial with 70 % ethanol and wipe off the excess ethanol. Take the cryovials to the laminar flow hood and remove the cap from each vial using sterile techniques. Using a P200 pipetman, add 100 ml of the adenoviral glycerol stock to 1000 ml of DMEM/1% PS (there will be two individual 1000 ml of DMEM/1% Penicillin/Streptomycin bottles). Thoroughly mix the 1000 ml of virus/media by gently swirling the bottle.

5. Using a sterile pasteur pipette, aspirate the media from each tissue culture plate and add 10 ml of virus/media to each tissue culture plate (MOI=10) using a 10 ml sterile pipette. There will be a total of 200 X 15 cm tissue culture plates This is done in a laminar flow hood using sterile technique.

6. Place the above tissue culture plates in a 37° C, 5 % CO_2 incubator for 1 hour.

7. Remove the tissue culture plates from the incubator and place in a laminar flow hood. Using a 25 ml sterile pipette, add 20 ml of DMEM/15% FBS/1%PS to each of above 15 cm sterile tissue culture plates.

8. Place the above tissue culture plates in a 37° C, 5 % CO_2 incubator for 36-40 hours or till the appearance of cytopathic effect (CPE), in which the adherent cells are round up due to the replication and assembly of adenovirus in the cells.

9. Remove the tissue culture plates from the incubator and place in a laminar flow hood. Using a 25 ml sterile pipette, pipet cells off the tissue culture plate and transfer the 30 ml of cell suspension from each tissue culture plates to a1000 ml sterile centrifuge bottle (750 ml cell suspension/bottle, a total of eight bottles).

10. Place each 1000 ml centrifuge bottle in a Beckman J6-HC centrifuge and spin down the cell pellet at 4000 rpm for 10 min at 4 °C.

11. Remove the bottles from the centrifuge and bring to the laminar flow hood. Using a sterile pasteur pipette, aspirate the supernatant from the bottles. Using a 10 ml sterile pipet, add 10 ml of 10 mM Tris-HCl, pH 8.1 to each bottle, resuspend the cell pellet by gently mixing the cell suspension, transfer the resuspension to a labeled 15 ml sterile test tube (a total of eight test tubes).

2. Place the above cell aliquots at -80 °C.

3. *Standard Operating Procedure (SOP) for Purification of Adenoviral DNA from Infected 293 Cells*

1. Make CsCl solutions using the above mentioned 10 mM Tris-HCl, pH8.1 solution:

   a)Heavy CsCl solution: 42.23 g CsCl in 57.77 ml of 10 mM Tris-HCl, pH 8.1 (density=1.45 g/ml).
   b)Light CsCl solution: 22.39 g CsCl in 77.61 ml of 10 mM Tris-HCl, pH 8.1 (density=1.20 g/ml).

2. Remove eight cell suspension aliquots (see SOP for the infection of 293 cells with adenoviral DNA) from -80 °C freezer and thaw in a 37 °C water bath followed by freezing and thawing two times in alcohol-dry ice/37 °C water bath to release virus from the cells.

3. Place each aliquot of cell lysates in a table top centrifuge and spin down the cell pellet using setting 8 for 10 minutes at 4 °C.

4. Remove the test tubes from the centrifuge and bring to the laminar flow hood. Using a 10 ml sterile pipette, transfer 10 ml of supernatant to a 50 ml sterile test tube. Using a 10 ml sterile pipet, add 8 ml of 10 mM Tris-HCl, pH 8.1 to each test tube, resuspend the cell pellet, repeat step 3, pool the 8 ml of supernatant with the original (a total of 18 ml for each aliquot) and place test tubes on ice.

5. Put the 18 ml of supernatant onto a SW28 ultracentrifuge tube containing 20 ml of CsCl gradient solution made with equal volumes of heavy (bottom layer) and light (top layer) CsCl solutions. There will be a total of eight tubes.

6. Place the SW28 tubes in a SW28 rotor and spin for 2 hours at 20,000 rpm, 4 °C.

7. Remove the tubes from the rotor, collect 2 ml of the virus band by puncturing the tube with a 18 gauge needle and 3 ml syringe and put into a 15 ml sterile tube.

8. Add 2 ml of 10 mM Tris-HCl, pH 8.1 to each tube and relayer 4 ml of viral solution onto a SW41 ultracentrifuge tube containing 8 ml of CsCl gradient solution made with equal volumes of heavy and light CsCl solutions.

9. Place the SW41 tubes in a SW41 rotor and spin for 12 hours at 20,000 rpm, 4 °C.

10. Collect 0.5 ml of virus band by puncturing each tube with a 18 gauge needle and 1 ml syringe, put into a 1 ml sterile vial and place it on ice.

11. Set up a 5 ml bed volume of Sephadex G-50 column and equilibrate the column with 5 bed volumes of PBS in the laminar flow hood. To remove CsCl from viral solution, load the 0.5 ml of viral solution onto the column followed by collection of about 1 ml of the virus fraction. Pool eight preparations of virus (~8 ml) and add 0.9 ml of glycerol to the final concentration of 10%.

12. Remove 20 ml of glycerol virus solution, dilute to 1 ml in water and measure the OD_{260} to determine the virus yield (OD_{260} X dilution X 10^{12} = particles/ml). In addition, save 10 ml for titering of virus solution by plaque assay and store the rest at -80 °C.

4. *Standard Operating Procedure for the Detection of Wild-Type Adenovirus - Helper virus assay #1.*

Preliminary studies to develop a standardized assay for the presence of wild type or helper adenovirus in our recombinant adenoviral stocks have been done using HeLa, a human cervical adenocarcinoma cell line, or A549, a human lung carcinoma cell line. The "wild type" strain DL7001 of adenovirus type 5 was used as a positive control. Cells were grown in Dulbeco's modified eagle media (DMEM), 10% fetal bovine sera (FBS), and 1% penicillin/streptomycin (P/S) (Gibco) in a standard 37°C CO_2 incubator.

1. 24 hours prior to initiation of the assay, cells were split into 10 cm tissue culture dishes at a density of 1 x 10^5 cells per dish.

2. The assay was begun by replacing the normal growth media with DMEM containing 1% P/S.

3. The plate is then inoculated with either no recombinant virus or a known pfu/cell of recombinant adenovirus, mixed with 0, 1, 10, 100, 1000 or 10000 particles per plate of purified DL7001 (wild type) adenovirus (based on O.D. at 260). Cells were then placed in a 37°C CO_2 incubator.

4. 2 hours later one ml of FBS was added to each plate to give a 10% final concentration. Cells were returned to the incubator and grown overnight.

5. The following day the virus containing media was replaced with fresh DMEM, 10% FBS, and 1% P/S. Cells were monitored daily and grown until full cytopathic effect (CPE) was noted. Media was replaced every 2 - 3 days.

Initial experiments were done with HeLa cells. In them it was determined that even in the absence of wild type (DL7001) virus, CPE was seen if more then 10 -100 pfu/cell of recombinant adenovirus was added to a plate. Subsequent reconstitution experiments were performed with recombinant virus at a MOI=10. In these experiments we were able to detect 1-10 particles of wild type virus (0.1 to 1 pfu) mixed with 10^6 pfu of

recombinant virus. The presence of wild type virus manifested as CPE on the indicator cell. It took 3 - 4 weeks to see full CPE at the highest dilution of virus.

Recently, in an attempt to allow for a more rapid assay as well as develop an assay which allowed higher concentrations of recombinant adenovirus to be present in an individual assay, we have used A549 cells. It was determined the A549 cells could be inoculated with up to 10,000 pfu/cell of recombinant virus without detectable CPE after 3 weeks in culture. We have now attempted an extension of this experiment in which 10^6 pfu (10 pfu/cell) and 10^9 pfu (1000 pfu/cell) recombinant virus/plate was mixed with 0, 1, 10, 100, and 1000 particles/plate of wild type virus (note: average pfu to particle ratio is 1/10). All dilutions of wild type virus (except 0) when in the presence of 1000 pfu/cell of recombinant virus exhibited CPE within one week of the initiation of the experiment. Similar sensitivity of wild type detection was achieved with reconstitution experiments using 100 pfu of recombinant virus/cell except that the development of CPE was delayed to three weeks.

D. Budget for the Certification of Recombinant Adenoviral Reagents

V. Facilities

To accommodate the need for gene therapy clinical activities, the University of Pennsylvania Medical Center has committed to renovating and equipping a 2500 sq. ft. laboratory, the Human Applications Laboratory (HAL). This space was completed in April 1993 and the construction drawings can be found in section V.B. It is equipped to do state-of-the art research and development in the arena of gene therapy. The laboratory operates under Good Laboratory and Good Manufacturing Practices (GLP and GMP) in accordance with guidelines and regulations established by the FDA. The objective of the design was to provide maximum utilization of the space for multiple clinical protocols. Therefore, the laboratory consists of a main area where general molecular biology experiments can be performed (i.e., cloning of new vectors, sequencing, centrifugations and storage of supplies and cells) as well as five separate tissue culture facilities that line the perimeter of the main lab. These tissue culture facilities are self-sustaining rooms which contain a biological laminar flow hood, two double-stacked CO_2 incubators, one refrigerated tabletop centrifuge, a fluorescent microscope and a benchtop containing a sink area. Each tissue culture area has its own air supply and return and is pressure-regulated so that it is positive to the main lab. The advantage of having these complete and separate tissue culture facilities is that it allows multiple gene therapy protocols to be in operation at the same time. We will be able to produce recombinant retroviral reagents in one room and at the same time produce recombinant adenoviral reagents in another room. It also allows for the harvest and isolation of cells from patients and growth of the cells in a separate room where no recombinant reagents have ever been produced. The cost of renovating this space and the major equipment was approximately $2.1 million dollars. A list of the major equipment needed to furnish the space is provided below. There are numerous supplies and small equipment items that still need to be purchased to make this a functional laboratory.

A. Budget Costs for Equipping a 2500 sq. ft. laboratory

Equipment	Vendor	Quantity	Price
1. Sterilizer	Getinge	1	79,350.00
Installation Type A	Getinge		5,100.00
Preventative Maintenance	Getinge		2,900.00

A Phase I Trial of the Administration of Autologous Breast Cancer Cells Transfected with B7 To Breast Cancer Patients As a Vaccine to Induce Immunity Against Breast Cancer

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INTRODUCTION:

The American Cancer Society estimates that there will be 182,000 new cases of female breast cancer and 46,000 deaths from breast cancer in 1993(1). Between 1980 and 1987 the age-adjusted incidence rate of breast cancer in the United States grew from 84.8/100,000 to 112.4/100,000, an increase of 32.5%(2). Much of this increase was attributed to the greater use of screening mammography and earlier detection of breast cancer. It appears that the breast cancer incidence rate is declining since 1987 with an incidence of 109.6/100,000 in 1988 and 104.6/100,000 in 1989 according to data from the National Cancer Institute's Surveillance, Epidemiology, and Results Program (SEER)(3).

Although there is hope that earlier detection will result in decreased mortality from breast cancer, so far, there has been no change in mortality rates according to SEER program data between 1973 and 1989. In fact, breast cancer mortality has changed little since 1930(1). Efforts to prevent the development of breast cancer through diet or other agents, as well as efforts to increase the earlier detection of breast cancer will be very important in the fight to decrease the incidence and mortality of breast cancer. However, many women will continue to be diagnosed with breast cancer and have spread of their disease beyond the breast either at the time of diagnosis or sometime later. Despite improvements in the adjuvant therapy of breast cancer, 20-40% of women with stage II breast cancer will eventually relapse and die of their disease(4). Once breast cancer metastases become clinically detectable, the disease will ultimately claim the life of the patient in spite of the fact that it often responds to chemotherapy treatments.

Additional modalities of treatment for advanced breast cancer are clearly needed. In addition to the standard modalities of surgery, radiation, chemotherapy and hormonal therapy, modulation of the immune system is another possible way of attacking breast cancer. Over the last fifteen years, dramatic discoveries have: enabled scientists to clone and produce many heretofore uncharacterized products of the immune system, increased the understanding of the immunobiology of cancer, and enhanced the fundamental knowledge of how cells in the immune system recognize antigens. This protocol is an effort to capitalize on these advances in an attempt to develop a new strategy to treat breast cancer.

Evidence from clinical studies conducted in patients with melanoma indicates that tumor regression induced by interleukin-2 can be mediated by cytotoxic T lymphocytes that are activated in vivo or adoptively transferred after ex vivo culture of tumor biopsies supplemented with interleukin-2

(these lymphocytes have been named tumor-infiltrating lymphocytes or TIL)(5,6). The response to interleukin-2 and TIL therapy is correlated with the number of TIL cells administered to the patient and to the specific cytotoxicity of the TIL cells for autologous tumor in vitro(6,7). In summary, it appears that the induction of a strong, specific cytotoxic T lymphocyte response in vivo to the tumor is important in mediating tumor regression.

The goal of this clinical trial will be to test an innovative method of inducing a strong and specific cytotoxic T lymphocyte response in vivo by immunizing breast cancer patients with autologous breast cancer tumor cells that have been transfected with the human B7 gene. The autologous breast cancer cells containing the human B7 gene will express the B7 molecule on their surface. The B7 molecule, normally found an antigen presenting cells, has recently been discovered to play a critical role in delivering a second co-stimulatory signal to T cells during antigen presentation(8,9,10). That is, in addition to the presentation of antigen to the T cell by the peptide/MHC complex, a second co-stimulatory signal is necessary to activate the T cell. In the case of CD4+ helper T cells, full activation leads to proliferation and the production of immunologically active cytokines that orchestrate an immune response(11,12,13,14,15). In the case of CD8+ cytotoxic T cells, co-stimulation is necessary for development of cytolytic capacity(16,17). In the absence of co-stimulatory signals, (for example, if B7 is not present on the antigen presenting cell) then, in certain circumstances, T cell tolerance can be created during antigen presentation(18,19). This mechanism could explain why many cancers do not elicit an immune response- because they lack the B7 molecule on the surface of their cancer cells- and they deliver a tolerogenic signal instead of an activating signal to the T cells.

B7 is a transmembrane glycoprotein with two immunoglobulin-like extracellular demains(20). It has a molecular mass of 30 kd and is a member of the immunoglobulin gene superfamily. B7 has two ligands on T lymphocytes, CD28 and CTLA4. CD28 is constitutively expressed on 95% of CD4+ T cells and 50% of CD8+ T cells(21,22). CTLA4 is expressed on T cells after activation(23). While the precise physiologic role of CTLA4 is not known, it is clear that the binding of B7 to CD28 is sufficient to provide the co-stimulatory signals needed for complete T cell activation(24).

Three different groups of investigators have conducted animal experiments that demonstrate an important role for B7 in inducing antitumor immunity in vivo. Townsend and Allison transfected a K1735 murine melanoma cell line with murine B7 and injected it into syngeneic mice(25). Compared to a control vector-transfected K1735, the B7 transfected tumor grew less well and was able to protect the host mice from a subsequent challenge of the parental tumor cell line. The effect

appeared to be dependent on the presence of CD8+ T lymphocytes, because depletion of this subset decreased the immunogenicity of the B7 transfected cell line and restored the growth rate of the tumor to that observed with the parental cell line or the vector-transfected control tumor cell line. Depletion of CD4+ T cells did not diminish the immunogenicity of the B7 transfected tumor cell line. Hellstrom and colleagues used the K1735 cell line that was further modified by insertion of a strong tumor antigen, the E7 gene product of human papilloma virus(26). They transfected this modified cell line with B7 and showed that immunization with the B7 transfected cell line protected animals from subsequent challenge of the parental tumor cell line. They also demonstrated that immunization with the B7 transfected cell line delayed the growth of a simultaneously placed non B7-transfected cell line that was in the opposite leg. The antitumor activity seemed to be mediated by CD8+ T lymphocytes based on depletion experiments. An important finding in their experiment was that immunization with the B7 transfected cell line induced specific CTL activity against not only the B7 transfected tumor cells but also the tumor cells that were not transfected by B7, indicating that it may not be necessary to transfect all of the tumor cells with B7 to elicit an immune response against the established tumor. These investigators reported that B7 transfection alone did not affect the growth of the K1735 murine melanoma cell line in this syngeneic host, however tumor size was examined only at one early time point (on day 21) and had the animals been observed at a later time period the tumor may have been shown to be regressing as it was in the previous study by Townsend and Allison. Glincher and colleagues demonstrated that mouse sarcoma cells genetically engineered to provide the co-stimulatory T-cell activation signal stimulated potent tumor-specific CD4+ T cells that caused rejection of both transfected and native neoplastic cells(27). In summary, these studies demonstrate that alteration of cancer cells by transfection with B7 results in an antigen specific CD8+ cytotoxic T lymphocyte or CD4+ helper T cell response. This response is important in decreasing the growth of not only the genetically transfected tumor but also the native tumor as well as creating immunity to future challenges with the tumor.

It is recognized that this manipulation alone may not be sufficient to produce regression of established advanced tumors in patients. Additional therapy such as administration of cytokines to expand the number of activated T lymphocytes, or harvest and in vitro expansion followed by adoptive transfer of tumor infiltrating lymphocytes T lymphocytes may be necessary to cause established tumors to regress. However, it is felt that immunizing with B7 transfected cancer cells represents a significant advance in eliciting a CTL response which is a necessary first step for an immune based therapy of cancer.

The underlying assumption of this strategy to treat breast cancer is that breast cancer cells have unique, tumor specific antigens that T cells recognize; however, they don't become fully activated because the antigens are presented without costimulation. Recent evidence indicates that patients with breast cancer can elaborate cytotoxic T lymphocytes that recognize epitopes on cell-surface mucin proteins that are aberrantly underglycosylated in breast cancer cells(28,29,30). Breast cancer cells from several patients have been tested and all have been shown to express no B7 on their surface (Stephen Eck, personal communication). However, breast cancer cells from patients to express the necessary MHC Class I and II molecules necessary to present antigens to CD4+ helper T cells and CD8+ cytotoxic T cells, respectively(31) and (Stephen Ethier, personal communication)

Certain obstacles stand in the way of using autologous B7 transfected breast cancer cells for a vaccine. Human breast cancer cells are notoriously difficult to grow in primary culture. Even if a cell line is obtained from a patient, the cells multiply slowly making retroviral transfection difficult. Finally, it is possible that the cell line established represents the selective expansion of only a subset of cancer cells that may not be representative of the primary tumor especially with regard to tumor antigen expression. Therefore, our approach will be to use fresh autologous breast cancer cells that have been transduced with the gene for human B7 using an adenoviral vector. The advantage is the ability to transduce nonproliferating cells and can result in a high level of expression of B7. Although the expression of the delivered B7 gene may be transient, we expect it will be present long enough to generate an immune response.

PREPARATION OF THE RECOMBINANT ADENOVIRUS (AD 5 SEROTYPE) EXPRESSING HUMAN B7

The preparation of this virus is described in the accompanying documents in appendix B. In brief, replication defective adenoviral vectors expressing human B7 (hB7) will be prepared as follows. We cloned the hB7 cDNA by RT-PCR using mRNA from Daudi cells and primers designed to amplify the entire protein-coding domain based on the published sequence. After cloning, the cDNA was sequenced to insure that it encoded normal human B7 and that no mutations had been introduced during PCR. The hB7 cDNA was excised from the plasmid as blunt-ended fragments and ligated into the pAdCMV-*l*acZ plasmid after removing the *lacZ* sequence with Not I and blunting the plasmid ends with the Klenow fragment of DNA polymerase I. After determining subclones with the correct orientation of the B7 cDNA by restriction digestion, we obtained pAdCMVhB7, that will be used to prepare recumbinant adenoviruses. The adenovirus vector will be generated by homologous recombination between pAdCMVhB7 and sub360 adenoviral DNA

(E1a$^-$ and E3$^-$,) in 293 cells (E1a$^+$). Recombination between linearized pAdCMVhB7 and sub360 adenoviral DNAs will generate a defective adenovirus genome in which the B7 cDNA, under the control of the CMV immediate-early promoter, replaces the adenovirus E1a gene. This defect will be complemented in *trans* in cells that express a transfected E1a gene (e.g. 293 cells) to generate replication-defective adenovirus virions. Specifically, NheI-linearized pAdCMVhB7 plasmid DNA and XbaI/ClaI-digested sub360 DNA will be co-precipitated with calcium phosphate and used to transfect 293 cells in serum-free media. Non-linearized sub360 DNA will be transfected as a positive control for transfection and adenovirus generation, and linearized sub 360 DNA will be transfected alone (without B7 plasmid DNA) as a background control for transfection and adenovirus generation in the absence of recombination. The following day, the cells are overlaid with 1.6% agar in media. The overlay is repeated on day 5, and day 9 plaques (focal areas of low cell density and lysed cells, indicative of spreading adenovirus infection) are visualized by neutral red staining. Individual plaques are harvested and used to infect fresh plates of 293 cells. After two rounds of plaque purification, the virus will be quantitatively expanded on multiple plates of 293 cells and purified by CsCl gradient centrifugation. The ability of the recombinant adenovirus to express B7 will be assayed by testing their ability to confer B7 surface expression on Hela cells (normally B7-) after a two hour exposure to virus and overnight incubation. This work will be completed in the Human Applications Laboratory of the Institute for Human Gene Therapy, where adenovirus preparations are routinely performed under condition of "good laboratory practice" and "good manufacturing practice". Appropriate tests will be performed to ensure that the virus prepared for human use will meet the criteria for FDA certification. (See accompanying documents for details). This procedure has previously been carrier out for another recombinant adenovirus which is currently in clinical trials.

OBJECTIVES:

1. To determine the toxicity of intradermally injected irradiated autologous breast cancer cells that have been transfected with the human gene for B7 in patients with advanced or metastatic breast cancer.

2. To determine the maximum number of transfected breast cancer cells that can be safely given to these patients.

3. To determine if the vaccination results in an immune response and to characterize that immune response at the vaccination site, in the draining lymph nodes and in the peripheral blood.

4. Observe patients for any antitumor responses.

ELIGIBILITY REQUIREMENTS:

1. Patients must have advanced breast cancer that has failed to respond to at least two standard chemotherapy regimens used in the metastatic setting and who are considered unlikely to benefit from further salvage chemotherapy regimens or hormonal regimens.

2. Patients must have a source of autologous tumor that can be easily harvested. This includes patients with subcutaneous or cutaneous metastases, patients with easily excisable lymph nodes containing metastatic tumor, and patients with malignant pleural effusions or acities.

3. Karnofsky performance and status equal to or greater than 70%.

4. Age equal to or greater than 18 years.

5. Life expectancy of at least three months.

6. Patients must have evaluable or measurable disease in addition to the disease that will be surgically removed for the purposes of formulating the autologous vaccine.

7. Adequate baseline hematopoietic function: platelet count equal to or greater than 100,000/mm^3, total white blood count equal to or greater than 3,000/mm^3, absolute granulocyte count greater than 1,500/mm^3, and absolute lymphocyte count greater than 500/mm^3, and absolute lymphocyte count greater than 500/mm^3.

8. Adequate pretreatment organ function: creatinine equal to or less than 1.5/mg/dl, bilirubin equal to or less than 1.5/mg/dl, SGOT equal to or less than 2.5 x upper limit of normal total calcium equal to or less than 11.0/mg/dl, PT less than 14 seconds, PTT less than 40 seconds.

9. If patients have had recent surgery, they must be fully recovered from the effects of that surgery.

10. Patients must not have received any antineoplastic chemotherapy for the four weeks preceeding entry onto the study (six weeks for nitrosoureas and mitomycin).

11. Patients must not have received irradiation for the four weeks prior to entry onto the study.

12. Ability to give informed consent.

EXCLUSION CRITERIA:

1. Patients who are anergic to recall antigens (i.e. <2 mm induration at 48 hours to all of the antigens in the CMI Multitest (Merieux Institute, Inc., Fla.)

2. Patients may not have received prior antitumor vaccines or immunotherapy (e.g. no prior IFN, TNF, IL-2, IL-4, IL-6, IL-1 or monoclonal antibodies).

3. History of any autoimmune diseases (e.g. SLE, rheumatoid arthritis, myasthenia gravis).

4. Evidence of HIV infection or AIDS.

5. Active infection (bacterial, fungal, or viral), or active bleeding (e.g. hemoptysis, GI bleeding).

6. Pregnancy or lactation; women of childbearing potential must use effective contraception during the course of this clinical trial.

7. Uncontrolled angina, arrhythmias, bronchospasm, hypertension, hyperglycemia or hypercalcemia.

8. No history of corticosteroid use in the four weeks preceeding entry onto the clinical study.

9. Patients who require corticosteroids are not eligible for this study.

10. Any medical or psychiatric illness which in the opinion of the protocol chairman would compromise the patients ability to tolerate this treatment.

11. Patients who require anticoagulation are not eligible.

12. There is no exclusion for sex or ethnic background.

INITIAL CLINICAL EVALUATION:

1. Complete history and physical exam.

2. CBC with differential and platelet count.

3. Serum chemistry profile.

4. PT and PTT.

5. Urinalysis.

6. HIV antibody, HBsAg titers.

7. Beta HCG in women of childbearing potential.

8. EKG.

9. PA and lateral chest x-ray.

10. Imaging studies as indicated in order to determine the extent of patient's metastatic disease and to follow it for response (within 3 weeks prior to the start of treatment)

11. Peripheral blood for baseline immune studies.

12. At the time of initial evaluation, the patient's ability to respond to recall antigens (tetanus, diptheria, streptococcus, tuberculin, candida, trichophyton and proteus antigens) will be tested with the Multitest CMI antigen applicator (Merieux Institute, Inc., Fla.) and the skin reaction will be read at 24 hours and 48 hours as per instructions of the manufacturer. Anergic patients will <u>not</u> be allowed to participate in this study.

13. Baseline tests to assess for evidence of the development of autoimmune disease: ANA, RF, CH50, anti-DNA abs, T4, TSH

STUDY DESIGN:

1. Surgery: Patients will undergo surgical removal of metastatic disease under local anesthesia in order to provide autologous tumor cells that can be transfected with the human B7 gene.

2. Vaccination preparation: A section of the removed tumor will be sent to surgical pathology for pathologic diagnosis. The remainder of the specimen will go to the laboratory to prepare B7 transfected autologous tumor and for immunologic assays. After the autologous breast cancer cells have been transfected with B7 they will be irradiated with 5000 rads a dose of radiation that renders them nontumorigenic but allows them to remain metabolically active. Cell viability will be tested by Trypan blue exclusion test.

3. Vaccinations: When the autologous transfected irradiated breast cancer cells are available they will be injected intradermally into the thigh approximately 10 cm below the inguinal lymph nodes and the site will be marked with and ink tatoo for future biopsy. The injections will be administered in the Clinical Research Center, University of Michigan Hospital and the patients will remain in the hospital over night.

4. Cohorts of six patients each will be treated with escalating doses of autologous irradiated B7 transfected breast cancer cells according to the following scheme (for technical reasons, 10^8 cells is the likely maximum number of cells that could be obtained from these patients):

   a. 10^6 cells
   b. 10^7 cells
   c. 10^8 cells

5. The injections will be given intradermally approximately 10 cm below the inguinal lymph nodes in the right or left anterior thigh. Patients at the highest dose of cells may require more than one injection.

6. Each patient will be observed for at least three weeks at a given level of cell injection before the patients are permitted to enroll on the next higher dose level of cells. If one or fewer patients experience dose limiting toxicity at a given number of cells injected, escalation will be permitted to continue to the next level. If two or more patients sustain dose-limiting toxicity, then that level of cells will be determined as the dose-limiting number of cells and the dose level of cells below that will be defined as the maximum tolerable dose of cells to be injected. It is possible that the maximum tolerated dose of transfected cells will not be observed because. for technical reasons, the concentration of cells/ml to be injected limits the escalation of cells to no greater than 10^8 cells.

7. Once escalation is completed, a separate cohort of six patients will be treated with both B7 transfected autologous irradiated breast cancer cells and vector alone transfected autologous irradiated tumor cells. One injection will be placed in the left thigh and one in the right thigh at the same time. The purpose of treating this cohort of patients is to compare the immunologic response at the vaccination site and in the draining lymph nodes from one leg to the other. This will help determine if B7 transfection enhances the immune response above that which is seen with transfection of vector alone.

8. A surgical biopsy of the vaccination site (or one of the vaccination sites) will be performed two weeks after the injection. Surgical removal of one to three draining lymph nodes will be performed approximately two weeks after the vaccination. Patients receiving both B7 transfected and vector alone transfected cells will receive injections into both thighs and these patients will have a biopsy of each vaccination site two weeks after vaccination These patients will also have one to three draining inguinal lymph nodes removed in both legs two weeks after vaccination.

9. Four weeks after vaccination the patients will undergo reevaluation to determine if their disease has responded or progressed. The patients whose disease has not worsened or has regressed (even if it does not meet the criteria for partial regression) will be eligible to receive additional cycles of treatment using the autologous B7 transfected irradiated cancer cells providing that they experienced no severe toxicity with the first

vaccination. Patients may continue this treatment until they have evidence of progressive disease.

IMMUNOLOGICAL MONITORING:

1. DTH testing: Patients will be tested for DTH responses by intradermal injections of both irradiated autologous transfected breast cancer cells and irradiated autologous nontransfected breast cancer cells at separate sites on the volar surfaces of both forearms. There will be three separate injections of the 10^4, 10^5, and 10^6 cells on both forearms. Induration will be assessed 24 and 48 hours later. DTH responses to all three doses of autologous tumor cells will be measured and recorded as the largest biperpendicular diameters of induration at 24 and 48 hour time points. A positive DTH test will be defined as induration measuring greater than 25 mm^2 overall (determined by multiplying biperpendicular diameters) as measured at 24 or 48 hours or both.

2. Biopsy of the vaccination injection site and the draining lymph nodes will be examined histologically for evidence of inflammation.

3. Peripheral blood lymphocytes will be assayed for changes in T cell subsets, and for evidence of proliferative response and cytokine production to, and lysis of autologous tumor.

4. Draining lymph nodes will be tested to determine if they contain lymphocytes that proliferate in response to autologous tumor and are cytolytic. All these assays will be performed with autologous tumor that has not been transfected with B7 and autologous tumor that has been transfected with B7.

5. Serological responses: Serum will be obtained to test for the development of antibodies to autologous breast cancer tumor cells and to autologous breast cancer cells that have been transfected with B7.

RESPONSE CRITERIA:

Standard response criteria will be used. Complete remission equals disappearance of all tumor for a period of one month. Partial remission equals a 50% or greater decrease in the sum of the products of the perpendicular diameters of all measured lesions without any evidence of

progression of any lesion or the apperance of any new lesion for a period of one month. Stable disease equals any change in measurable disease which is less than the criteria for partial remission or progression without any evidence of new lesions. Progressive disease equals a 50% or greater increase in the biperpendicular product of any single lesion, the development of any new lesion, or the significant clinical deterioration related to the progression of patients disease.

CRITERIA FOR DISCONTINUATION OF THE TREATMENT:

1. Unacceptable toxicity.

2. Intercurrent illness which prevents further administration of treatment.

3. Patient preference.

4. Progressive disease.

TOXICITY:

The major toxicity that is anticipated will be local and this includes redness, swelling, pain, and increased warmth at the injection site. Patients will be monitored for the development of clinical symptoms suggesting autoimmune disease or allergic reactions.

Toxicity will be graded according to the Cancer Treatment Evaluation Program toxicity scale (see appendix A). Local toxicity will be graded as follows: erythema and induration less than 20 mm equals grade 1; erythema and induration greater than 20 mm but no ulcer equals grade 2; ulcer or painful regional adenopathy equals grade 3; and permanent dysfunction related to local toxicity equals grade 4. Patients will not be considered to have dose limiting local toxicity unless they have grade 4 toxicity. All other grade 3 or grade 4 toxicity is considered dose limiting and patients will be removed from study if they experience it.

REPORTING ADVERSE DRUG REACTIONS:

Adverse drug reactions will be reported to the Human Use Review and Regulatory Affairs Office of the USARMDC.

SERIAL STUDIES:

Serial studies (PE,cbc,chem panel,PT,PTT,UA, and tests for autoimmune disease) will be performed weekly during the first month to assess toxicities. In addition, sera and peripheral blood mononuclear cells will be obtained for archival purposes according to the current safety monitoring guidelines by the Center for Biologics Evaluation and Research (presently, once/month on treatment and every three months thereafter). In addition, the following table describes the investigational studies that will be performed:

	Pretx	2 wks	4 wks	8 wks	12 wks
DTH			X	X	X
Skin & LN Biopsies		X			
T cell subsets	X	X	X	X	X
Antibody screening	X	X	X	X	X
In vitro T cell assays	X	X	X	X	X

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COMMON TOXICITY CRITERIA

TOXICITY	GRADE 0	1	2	3	4
BLOOD/BONE MARROW					
WBC	≥4.0	3.0 – 3.9	2.0 – 2.9	1.0 – 1.9	<1.0
PLT	WNL	75.0 – normal	50.0 – 74.9	25.0 – 49.9	<25.0
Hgb	WNL	10.0 – normal	8.0 – 10.0	6.5 – 7.9	<6.5
Granulocytes/ Bands	≥2.0	1.5 – 1.9	1.0 – 1.4	0.5 – 0.9	<0.5
Lymphocytes	≥2.0	1.5 – 1.9	1.0 – 1.4	0.5 – 0.9	<0.5
Hemorrhage (clinical)	none	mild, no transfusion	gross, 1–2 units transfusion per episode	gross, 3–4 units transfusion per episode	massive, >4 units transfusion per episode
Infection	none	mild	moderate	severe	life-threatening
GASTROINTESTINAL					
Nausea	none	able to eat reasonable intake	intake significantly decreased but can eat	no significant intake	—
Vomiting	none	1 episode in 24 hrs.	2–5 episodes in 24 hrs.	6–10 episodes in 24 hrs.	>10 episodes in 24 hrs., or requiring parenteral support
Diarrhea	none	increase of 2–3 stools/day over pre-Rx	increase of 4–6 stools/day, or nocturnal stools, or moderate cramping	increase of 7–9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, or ulcers, and cannot eat	requires parenteral or enteral support
LIVER					
Bilirubin (BRMP)	WNL WNL	— <2.5 x N	<1.5 x N 2.6–5 x N	1.5 – 3.0 x N 5.1 – 10 x N	>3.0 x N >10 x N
Transaminase (SGOT, SGPT)	WNL	≤2.5 x N	2.6 – 5.0 x N	5.1 – 20.0 x N	>20.0 x N
Alk Phos or 5′ nucleotidase	WNL	≤2.5 x N	2.6 – 5.0 x N	5.1 – 20.0 x N	>20.0 x N
Liver — (clinical)	no change from baseline	—	—	precoma	hepatic coma

COMMON TOXICITY CRITERIA (Continued)

TOXICITY	GRADE 0	1	2	3	4
KIDNEY, BLADDER					
Creatinine	WNL	<1.5 x N	1.5 – 3.0 x N	3.1 – 6.0 x N	>6.0 x N
Proteinuria	no change	1+ or <0.3 g% or <3 g/l	2 – 3+ or 0.3 – 1.0 g% or 3 – 10 g/l	4+ or >1.0 g% or >10 g/l	nephrotic syndrome
Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion
Alopecia	no loss	mild hair loss	pronounced or total hair loss	—	—
Pulmonary	none or no change	asymptomatic, with abnormality in PFT's	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
Symptoms (BRMP)	none or no change	mild	exertional dyspnea	dyspnea at rest; requires intermittent O_2	requires continuous O_2 or assisted ventilation
Function (BRMP)	none or no change	25–50%↓ in DLCO or VC	>50%↓in DLCO or VC	—	—
HEART					
Cardiac dysrhythmias	none	asymptomatic, transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia, or fibrillation
Cardiac function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac—ischemia	none	non-specific T-wave flattening	asymptomatic, ST and T-wave changes suggesting ischemia	angina without evidence for infarction	acute myocardial infarction
Cardiac—pericardial	none	asymptomatic effusion, no intervention required	pericarditis (rub. chest pain, ECG changes)	symptomatic effusion: drainage required	tamponade: drainage urgently required
BLOOD PRESSURE					
Hypertension	none or no change	asymptomatic, transient increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	recurrent or persistent increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	requires therapy	hypertensive crisis
Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement of other therapy but not hospitalization	requires therapy and hospitalization; resolves within 48 hours of stopping the agent	requires therapy and hospitalization for >48 hours after stopping the agent

COMMON TOXICITY CRITERIA (Continued)

OXICITY	GRADE 0	1	2	3	4
IEUROLOGIC					
Ieuro— sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss: moderate paresthesias	severe objective sensory loss or paresthesias that interfere with function	—
Neuro— motor	none or no change	subjective weakness: no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro— cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neuro— cerebellar	none	slight incoordination, dysdiadokinesis	intension tremor, dysmetria, slurred speech, nystagmus	locomotor atoxia	cerebellar necrosis
Neuro— mood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro— headache	none	mild	moderate or severe but transient	unrelenting and severe	—
Neuro— constipation	none or no change	mild	moderate	severe	ileus >96 hours
Neuro— hearing	none or no change	asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro— vision	none or no change	—	—	symptomatic subtotal loss of vision	blindness
Skin	none or no change	scattered macular or papular eruption or erythema that is asymptomatic	scattered macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Edema (BRMP)	none or no change	mild	moderate	severe	—
Allergy	none	transient rash, drug fever <38c, 100.4F	urticaria, drug fever =38c, 100.4F mild bronchospasm	serum sickness, bronchospasm, req parenteral meds	anaphylaxis
Fever in absence of infection	none	37.1 - 38.0c 98.7 - 100.4F	38.1 - 40.0c 100.5 - 104.0F	>40.0c >104.0F for less than 24 hours	>40.0c (104.0F) for more than 24 hrs. or fever accompanied by hypotension

COMMON TOXICITY CRITERIA (Continued)

	GRADE				
OXICITY	0	1	2	3	4
ocal	none	pain	pain and swelling, with inflammation or phlebitis	ulceration	plastic surgery indicated
'eight ;ain/ loss	<5.0%	5.0 - 9.9%	10.0 - 19.9%	≥20.0%	—
ETABOLIC					
·yperglycemia	<116	116 - 160	161 - 250	251 - 500	>500 or keto-acidosis
Hypoglycemia	>64	55-64	40 - 54	30 - 39	<30
Amylase	WNL	<1.5 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	>5.1 x N
Hypercalcemia	<10.6	10.6 - 11.5	11.6 - 12.5	12.6 - 13.5	>13.5
Hypocalcemia	>8.4	8.4 - 7.8	7.7 - 7.0	6.9 - 6.1	≤6.0
Hypomagnesemia	>1.4	1.4 - 1.2	1.1 - 0.9	0.8 - 0.6	≤0.5
COAGULATION					
Fibrinogen	WNL	0.99 - 0.75 x N	0.74 - 0.50 x N	0.49 - 0.25 x N	≤0.24 x N
Prothrombin time	WNL	1.01 - 1.25 x N	1.26 - 1.50 x N	1.51 - 2.00 x N	>2.00 x N
Partial thromboplastin time	WNL	1.01 - 1.66 x N	1.67 - 2.33 x N	2.34 - 3.00 x N	>3.00 x N

INFORMED CONSENT

A PHASE I TRIAL OF THE ADMINISTRATION OF AUTOLOGOUS BREAST CANCER CELLS TRANSFECTED WITH B7 TO BREAST CANCER PATIENTS AS A VACCINE TO INDUCE IMMUNITY AGAINST BREAST CANCER

I am being offered an opportunity to take part in a clinical research study at the University of Michigan Cancer Center, Ann Arbor, Michigan. Federal regulations require written informed consent prior to participation in a study using an investigational drug so that I can know the nature of risks of my participation and can decide to participate or not to participate in a free and informed manner. The following consent form describes the purpose, procedures, benefits, risks (side effects), discomforts, and precautions which go along with my participation. In addition, this consent form outlines my rights as a patient involved in such an investigational (experimental) protocol. I am being asked to read this document to insure that I am fully informed of the nature of this treatment protocol and of how I will participate in it if I consent to do so. I am being urged to discuss any questions I have about this study with the staff members who will explain it to me. Signing this form will indicate that I have been informed about this study and that I give my consent.

Funding for this study comes from the Department of the Army, U.S. Army Medical Research and Development Command. Neither the Army, the researchers, nor the doctors at the University of Michigan Hospital have a vested interest in either the procedures involved or the outcome of this study, and the funding provided to support this study by the Army does not depend on the outcome.

Background:

I have a diagnosis of breast cancer that has recurred and has been treated at least twice with standard anti-cancer drugs (chemotherapy) and/or hormonal therapy. Despite these treatments, my cancer continues to grow.

In addition to the standard treatments of surgery, radiation therapy, chemotherapy, and hormonal therapy that are designed to kill cancer cells, another modality of treatment called immunotherapy is being tested in cancer patients. Immunotherapy involves attempts to use the immune system or products of the immune system to fight cancer. Currently there is no FDA approved immunotherapy treatment for breast cancer. The immunotherapy that is part of this study is experimental.

One of the ways cancer patients have been treated in the past with immunotherapy is with an anti-tumor vaccine. This means that the patient's tumor (or another patient's tumor) is isolated and purified, then is treated with irradiation (so the tumor cells won't grow) and injected under the skin. The tumor cells are usually given with another agent called an adjuvant that is designed to stimulate the immune system resulting in recognition of the cancer cells by the immune system. It is hoped that this approach will then lead to destruction of other cancer cells elsewhere in the body by the cells in the immune system that can do this. Vaccines have been very effective in preventing many diseases caused by viruses or bacteria. To date, they have not been shown to be definitely beneficial in the treatment of cancer, although large clinical studies are currently testing anti-tumor vaccines in patients with colon cancer and melanoma (a type of skin cancer).

Recent scientific discoveries have lead to a better understanding of what the immune system requires in order to respond strongly to a tumor cell. It has been shown that the immune cells called lymphocytes (a type of white blood cell) require two signals in order to become fully

activated and acquire the ability to kill cancer cells. The first signal is the marker on the cancer cell (antigen) which is not contained on other cells in the body. The second signal is provided when a molecule called B7 interacts with its ligand (or receptor) on the lymphocyte resulting in the expansion of the number of lymphocytes that can recognize and kill the tumor cell. Recent experiments have indicated that tumor cells do not possess B7, the molecule that is necessary to deliver the co-stimulatory signal to lymphocytes. Because the tumor cell lacks the B7 molecule, when a lymphocyte interacts with it, the lymphocyte fails to become activated and in fact becomes "paralyzed" in its ability to respond to the cancer cell. Scientists now believe that this is an important way that cancer cells escape rejection by the immune system.

Experiments conducted in animals have shown that a cancer cell can be altered so that it expresses the B7 molecule. This alteration is accomplished by a process known as genetic engineering, a technology that involves the recombination of DNA (the molecules that make up genes). The gene that instructs the cell to make the B7 molecule is inserted (transfected) into a cancer cell resulting in the production and expression of this molecule on the surface of the cancer cell. In the animal experiments, the tumor cells that were transfected in such a fashion failed to grow when placed under the skin of a animal, whereas the tumor cells that were unaltered grew and formed a tumor. In some studies, the genetically altered tumor cells were capable of causing unaltered tumor cells to be killed by the immune system. It is on the basis of these experiments that this protocol has been designed. It is hoped that the alteration in the tumor cell created by the transfection of a gene for B7 will result in an enhanced recognition of the tumor by the immune system. It is not known at the present time whether this desired effect-enhanced recognition of the tumor by the immune system-will occur in humans, nor is it known whether the accomplishment of that goal will translate into a shrinkage of tumor in cancer patients with advanced breast cancer.

Purpose and Design of this Study:

This study is being conducted to determine the safety, side effects, and toxicity of a genetically altered breast cancer tumor cell vaccine. Another reason this study is being conducted is to determine if the vaccine causes an immune response in cancer patients. All patients will receive the genetically altered vaccine. A few patients will receive a vaccine of their own breast cancer cells without the gene for B7 in addition to the vaccine of their own breast cancer cells with the gene for B7. The purpose of treating this group of patients is to compare the immunologic response in these patients to the two different vaccines. Patients will receive different numbers of breast cancer cells injected under their skin starting with a lower number of cells and increasing to a higher number of cells as long as there are no severe side effects. Each individual patient will receive only one amount of breast cancer cells, that is, the number of breast cancer cells in the vaccine will not be escalated in a given patient.

Description of the Treatment and Other Procedures:

Pretreatment screening: If I decide to participate in this study I will first be screened to make sure I meet the eligibility criteria. The screening process is done to make sure that it is appropriate for me to participate in this study. This process will include a review of my medical history, a complete physical examination, standard blood tests, an EKG (an electrocardiogram), a chest x-ray and if it is possible for me to become pregnant, a pregnancy test. These tests are normally performed on patients prior to receiving chemotherapy for breast cancer. In addition to these tests which are normally performed, blood will be obtained for tests which are specific for this investigational study. These tests include a blood test to determine whether I have an infection with the hepatitis B virus, a blood test to determine whether I have an infection with the AIDS virus, and blood tests to determine the ability of my immune system to respond to normal stimulation. A doctor will also order other x-rays, CT scans, or nuclear medicine scans to measure the extent of my breast cancer because the study treatment has a possibility of shrinking my tumor and this will be determined by measuring my disease before and after treatment. At the time of this initial evaluation I will have a

skin test which will determine my ability to mount an immune response to infectious agents I have been exposed to. If I fail to demonstrate a response in this skin test, I will not be allowed to participate in this study because such lack of response indicates that my immune system is significantly depressed and therefore may not respond to the vaccine treatment planned in this study.

I should also know that before beginning treatment it is necessary to test my blood for antibodies to the human immunodeficiency virus (HIV), the virus that causes the Acquired Immune Deficiency Syndrome (AIDS). If I am found to have these antibodies in my blood stream I will not be able to participate in this experimental program because there is concern that the treatments that are administered on this study might alter the immune system in such a way as to worsen the viral infection. If I am found to have these antibodies, or if I have been diagnosed as having AIDS, I should be aware of the following policy: 1) my physician will notify me promptly of the result; 2) my physician will offer me any current and/or ongoing sexual partner(s) (spouses are generally considered to be current or ongoing sexual partner) or needle-sharing partner(s) I identify information on the meaning of the test and how to prevent the spread of this infection; 3) because HIV can be transmitted in several ways, it is important that I inform these partners that any or all may have been exposed to the HIV virus and encourage them to be tested. If I request, the University of Michigan will assist me in notifying partner(s) and arrange counseling; 4) in the event that I am unwilling or unable to notify partner(s), the University of Michigan is responsible for attempting to assure that they have been made aware of their possible exposure to HIV. All reasonable attempts will be made to protect my identity (for example, the partner(s) will be notified that they have been exposed to HIV without naming the individual who exposed them). Some of these notification and counseling procedures may be carried out through arrangements with, or referral to, the appropriate public health agency.

Vaccine treatment: In order to make a vaccine from my tumor I must have surgical removal of some tumor in order to provide tumor cells that can be genetically altered with the B7 gene. My doctor will determine if I have breast cancer cells that are easily obtainable. Examples would include fluid in my lung that contained breast cancer cells (known as a malignant pleural effusion), fluid in my abdomen which contained breast cancer cells (known as malignant ascites), tumor nodules in or directly under my skin, or tumor enlargement of lymph nodes that can be easily removed surgically. If the only way that my breast cancer cells could be obtained would require me to undergo general anesthesia, then I will not be able to participate in this study. All breast cancer cells obtained for the purpose of making the vaccine will be obtained using local anesthesia.

The breast cancer cells obtained from me will be taken to the laboratory where they will be transfected with the gene for the B7 molecule. This is accomplished by using a type of virus to integrate the gene into my breast cancer cells' DNA. The virus being used in this procedure is an adenovirus and it has been tested to make sure it cannot multiply in my body. After the breast cancer cells are transfected with the gene for the B7 molecule they will be irradiated (treated with x-rays) so that these cancer cells will die after a period of time. The breast cancer cells that have been genetically altered and irradiated will then be injected underneath my skin in my thigh. The injection will be done in the hospital and I will spend the night in the hospital to watch for any side effects from the injection.

Two weeks after the injection I will undergo a biopsy of the site where the breast cancer cells were placed under my skin. I will also undergo a minor surgical procedure to remove one to three lymph nodes from my groin. These tests are being done for research purposes to determine if the vaccine caused an immune response in my skin and my lymph nodes. At this time, my skin will be tested for the ability to respond to my breast cancer cells. Blood will also be drawn to determine if my circulating immune cells have developed the capacity to respond to my breast cancer cells. The skin testing and blood drawing are for research purposes.

Four weeks after my vaccination I will have the skin test and blood tests repeated. At this time I will also have x-rays and/or CT scans to determine whether my disease has responded to the vaccination treatment. If my breast cancer has not worsened or if it has shrunk to any extent, then I will be allowed to receive additional vaccinations using the genetically altered breast cancer cells, providing that I did not experience severe toxicity with the first vaccination. I may receive these cells injected under my skin once a month until there is evidence that my disease has started to grow or worsen. If I continue to receive the vaccine treatments, I will have the skin tests, and blood tests repeated once a month and the scans to measure my disease will be repeated once every two months.

The patients who receive two vaccines (the genetically altered breast cancer cells in one thigh and the cancer cells without the B7 gene in the other thigh) will have a treatment plan identical to that described above except that they will undergo a biopsy of both vaccination sites and undergo removal of one to three lymph nodes from both groins two weeks after their vaccination.

Post-Treatment Follow-up: Once my doctor determines that my disease is growing I will no longer be eligible to receive additional vaccine treatments. I may then receive other treatments for my breast cancer including experimental treatments as my doctor decides in conjunction with me. I will be required to come back to have my blood drawn every three months to test for the development of a reaction in my blood to the adenoviral vector. These blood tests are required by the FDA and are for the purpose of evaluating the safety of the vaccine treatment.

Blood Drawing: The maximum amount of blood that will be drawn during the first month will total 200 ccs which is approximately 40 teaspoons, which is equivalent to approximately 13 tablespoons over the four week time period. Thereafter, only 50 ccs of blood will be drawn per month, which is equivalent to 10 teaspoons (which is equivalent to approximately 3 tablespoons).

Risks

The treatment proposed for me in this study may involve certain side effects (toxicities). Side effects known to occur or which might occur are listed below. These are side effects that have been seen in other patients receiving vaccinations, although these other patients have not received the exact vaccination being proposed in this study. Any time a new treatment is given, there is the possibility of other side effects occurring which are not anticipated. The unanticipated side effects could be serious or possibly even fatal. It is important to tell my doctor of any reactions, problems, or side effects to the treatment.

It is possible that I may develop redness, swelling, increased warmth, tenderness, or pain at the site where the vaccine has been injected under the skin. It is possible that my skin may develop an ulcer at this site. The lymph nodes in my groin may become tender, painful, swollen, or reddened.

The minor surgical procedures performed to obtain breast cancer cells to make the vaccine will be performed using local anesthesia. Side effects from the local anesthetic are rare but can include minor to severe allergic reactions and cardiac irregularities.

It is theoretically possible that the vaccine treatment could cause my body's immune system to react against other cells in the body. The investigators in charge of the study will be testing my blood for any evidence of this and will monitor my symptoms to determine if you have evidence for such a reaction. It is also theoretically possible that you may have evidence for replication of the virus in your body that was used to transfect the breast cancer cells. Again, your blood will be tested for this.

The testing that you will have during therapy can also result in side effects. Blood testing is associated with the occasional risks of bruising or bleeding at the site from which the blood is

withdrawn. Very rarely, infection or clotting of the blood vessel can occur. The blood that is being removed for testing may worsen any anemia you may already have from the breast cancer or your previous chemotherapy, possibly making you feel more fatigued. It is also possible that you will require a blood transfusion for anemia, though this is thought to be unlikely given the amount of blood that will be withdrawn. If you require a blood transfusion you should be aware that these can be associated with the transmission of infection or can cause allergic reactions although both of these types of complications are quite unusual because of the extensive testing of the blood prior to transfusion.

In order to participate in this study, I should have avoided becoming pregnant from the first day of my most recent menses. I should avoid becoming pregnant for at least one year after my last vaccination. Pregnancy within one year after my last vaccination may create a potential risk to the unborn baby. To avoid becoming pregnant, I should either abstain from sexual relations or practice a method of birth control. Except for surgical removal of the uterus, birth control methods such as the use of condoms, a diaphragm or cervical cap, birth control pills, IUD, or sperm killing products are not totally effective in preventing pregnancy. The only ways to completely avoid risk to the unborn baby are 1) not to become pregnant or 2) not to participate in this study. Adverse effects might affect a developing fetus, and might result in unknown risks of deformities or death to the unborn baby. A negative pregnancy test does not absolutely prove that I am not pregnant. Regardless of the results of the pregnancy test which I was administered as part of the screening for this study, I should not participate in the study if I think that there is a possibility that I might be pregnant. If I become pregnant while participating in the study I must notify the principal investigator of the study, John W. Smith II, M.D. immediately. If I become pregnant while participating in this study I will be withdrawn from the study and not be allowed to receive other vaccinations.

Compensation for Illness or Injury:

I am authorized all necessary medical care for injury or illness which is the proximate result of my participation in this research. Contractors must provide such medical care when conducting research on private citizens. Other than medical care that may be provided (and any other remuneration specifically stated in this consent form), there is no compensation available for my participation in this research study; however, I understand this is not a waiver or release of my legal rights. I understand that the University of Michigan will provide full and prompt medical treatment in the event of physical injury resulting from research procedures. Additional medical treatment will be provided in accordance with the University's determination of its responsibility to do so. The University does not, however, provide compensation to a person who was injured while participating as a research subject nor does the University pay a patient for such participation.

My physician will be checking me closely to see if any of the side effects described above are occurring. Periodic physical exams, blood and urine tests, x-rays and/or scans will be performed to evaluate my disease status and to detect early any possible side effects. Many side effects disappear after investigational agents are stopped. In the meantime, my doctor may prescribe medications to keep these side effects under control. I understand that treatment to help control side effects could result in added cost. This institution is not financially responsible for treatment of side effects caused by the protocol therapy.

Treatment Duration and New Information:

The length of treatment on this study is a minimum of four weeks. If my physician determines that I am benefiting from this treatment, I may continue it on a monthly basis for an indefinite period of time so long as I continue to demonstrate clinical benefit from the treatment. During the study, should other treatment alternatives be clinically indicated, they will be discussed with me. Also, if

there are any significant new findings developed over the course of this research which may affect my willingness to participate, I will be provided with them.

Cost and Payments:

Clinic visits, laboratory or other tests, which are not being conducted for research purposes will not be free. These costs will be billed to me or my insurance carrier. How much I will have to pay depends on whether or not I have insurance and what cost my insurance covers. I will not be charged for the administration of the vaccine nor will I be charged for any of the investigation-related examinations or laboratory tests.

Benefits and Alternative Treatments:

In addition to the treatment being offered to me in this study the alternative treatments for my breast cancer include: 1) additional chemotherapy regimens; 2) additional hormonal therapies; 3) combinations of chemotherapy and hormonal agents; 4) investigational chemotherapy or hormonal agents; 5) other investigational immunotherapy treatments. The University of Michigan doctors are very willing to discuss the benefits and side effects of alternative treatments including other experimental therapies or the option of no further anti-cancer therapy. I am encouraged to ask questions and take as much time as I need to make my decision.

The purpose of this study is to evaluate the side effects and toxicity of a genetically altered breast cancer cell vaccine. It is not possible to predict ahead of time whether you will directly benefit from participating in this study; however, the possibility of benefit does exist. In addition, other patients may benefit in the future from the information gathered from this study. I understand that at least 24 patients will be treated on this study.

Right to Refuse and Withdrawl:

I may discontinue participation at any time without penalty or loss of benefit to which I am otherwise entitled. My physician may stop the protocol therapy if excessive side effects occur, if the vaccine is no longer available, or if I refuse treatment as recommended, refuse tests needed to determine safety or fail to return for recommended follow-up. However, unless I withdraw my consent, information will continued to be collected on my condition and I will still be expected to return for a follow-up visit to assess the safety of the study treatment and evaluate response.

Confidentiality:

I understand that my physician and the University will not identify me in any publication of this investigation and will keep records identifying me confidential to the extent provided by Federal, State, and local law. However I understand that during required reviews, representatives of the Food and Drug Administration, Department of Defense (e.g., the United States Army Medical Research and Development Command) may inspect my records. I consent to this review and also authorize the release of copies of my medical records, imaging studies, and laboratory/pathology specimens as necessary for evaluation of my disease and therapy.

In the past, studies involving genetic modification of cells have received a great deal of news coverage. Although these studies are more common today, it is still possible that the current trial may receive some attention by the news media. Under no circumstances will your identity or your participation in this study be revealed.

It is the policy of the US Army Medical Research and Development Command (USAMRDC) that data sheets are to be completed on all volunteers participating in research. For entry into USAMRDC's Volunteer Registry Data Base and the Volunteer Registry Data Base. The

information to be entered into the data base includes your name, address, social security number, study name, and dates. The intent of the data base is to: first, to readily answer questions concerning an individuals participation in research sponsored by USAMRDC; and second, to insure that the USAMRDC can exercise its obligation to insure that research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at USAMRDC for a minimum of 75 years. The computer data base will be kept confidential and not released to anyone.

Problems or Questions:

I should feel free to ask my University of Michigan physician about any of the details of the study treatment. The physicians involved in my care have been available to answer your questions about this treatment program and will continue to be available in the future. A copy of this consent form is kept on file and a copy will be given to you. You may call Dr. John W. Smith II, the protocol chairman, at 313-936-5281, with questions about the study or about any injury you feel is related to the study. If I have any questions regarding my rights as a research subject I may contact the chairman of the Institutional Review Board at the University of Michigan, Dr. William W. Coon at 313-936-5827.

VOLUNTARY CONSENT:

I have discussed the above information with Dr. _____ and have been given the opportunity to ask questions which have been answered.

( ) I have not engaged in any other research projects within the past six months.
( ) Within the past six months I have been involved in a study by Dr._____.
I have ( ) have not ( ) been under the care of a physician within the past 12 months. I agree to participate in this study. I have read and been given a copy of this consent form.

_____	_____
Patient's Name	Physician Name
_____	_____
Patient's Signature	Physician Signature
_____	
_____	
Patient's Address	
_____	_____
Witness Name	Date
_____	
Witness Signature	

EARLE A. CHILES RESEARCH INSTITUTE

JOHN W. SMITH II, M.D.
Chief, Clinical Research

TEL: (503) 215-3635
FAX: (503) 215-6841
email: john_smith@phsor.org

Appendix 2

January 18, 1997

Danny Laspe
US Army Medical Research Acquisition Activity
Attention: MCMR - AAA - A
Fort Detrick
Frederick, MD 21702-5014

RE: DOD Grant "Gene Therapy of Human Breast Cancer", DAMD17-94-J-4385, DRDA 941453

Dear Mr. Laspe:

The purpose of this letter is to notify you of some changes regarding the above referenced DOD grant. For the first two years of this grant, I served as the Principal Investigator; however, in August of 1996, I left the University of Michigan to assume my new position at the Earle A. Chiles Research Institute, Providence Portland Medical Center. In previous telephone conversations with you, I learned that the DOD grants are made to institutions and can't be taken away from that institution when the Principal Investigator leaves. After discussion with involved parties at the University of Michigan, it has been decided to name the following new Principal Investigator:

Laurence H. Baker, D.O.
Professor of Medicine and Associate Chief,
Division of Hematology/Oncology,
Department of Internal Medicine
University of Michigan School of Medicine
Director for Clinical Research and Deputy Director
University of Michigan Comprehensive Cancer Center
3119 Taubman Center
1500 E. Medical Center Drive
Ann Arbor, MI 48109-0374

Dr. Baker's biosketch and other support pages are enclosed for your review. The University of Michigan is in the process of establishing a subcontract with the Earle A. Chiles Research Institute to allow Dr. Smith to continue to help coordinate the research grant especially with the performance of tasks 3 and 4 in the statement of work, i.e. to

conduct a Phase I clinical trial of B7 transduced autologous breast cancer cells as a vaccine and to perform immunological monitoring studies on the patients participating in the trial. The joint enrollment of patients will be advantageous because the patient population (i.e. breast cancer patients with an easily obtainable source of autologous tumor) is relatively rare. Patient accrual will be improved by having both centers involved. Because the start of the clinical trial has been delayed by difficulty in manufacturing the adenoviral vector, there is less time to conduct the study and it would be extremely difficult for a single institution to complete the trial within the remaining time on the grant.

The Earle A. Chiles Research Institute, Portland Providence Medical Center was the first institution to conduct a gene therapy protocol in the state of Oregon. Currently, there are two active gene therapy studies at our institution, including a protocol for breast cancer patients, funded by the NIH, that is similar to the Army grant. Their proposal differs because it uses an HLA-A2 matched human breast cancer cell line instead of using autologous tumor, but both are transducing the breast cancer cells with B7 (CD80) to make the tumor immunogenic. Therefore, Dr. Smith and his colleagues at the The Earle A. Chiles Research Institute are completely capable and eminently qualified to conduct the clinical protocol and immunological monitoring as described in the statement of work. Dr. Smith will be in frequent contact with his collaborators and in addition, will make two trips per year to the University of Michigan for on site meetings.

In addition, we would like to request that some of the granted money be permitted to be spent on supporting the animal research that the original reviewers strongly suggested be performed before starting a clinical trial in patients with breast cancer.

Please let me know if there are any questions regarding these changes and whether any further action is required by any of the involved parties. Thank you in advance for your attention to this matter.

Sincerely,

John W. Smith II, M.D.

John W. Smith II, M.D.
Chief, Clinical Research

Encl.

CC: Laurence H. Baker
Alfred E. Chang
Stephen P. Ethier
Pam Stout
Walter Urba

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH ACQUISITION ACTIVITY
FORT DETRICK, FREDERICK, MD 21702-5014

REPLY TO ATTENTION OF

February 10, 1997

R&D Branch A

SUBJECT: Modification P70001 to DAMD17-94-J-4385
Principal Investigator: Laurence H. Baker, D.O.

RECEIVED
FEB 14 1997
N. D. GERL

Mr. Neil Gerl, Ph.D.
Project Representative
Division of Research Development
and Administration
The University of Michigan
3003 South State Street
Ann Arbor, Michigan 48109-1274

Dear Mr. Gerl

Enclosed is a fully executed original of the subject modification for your information and files.

If you have any questions, please contact the undersigned at (301) 619-7147.

Sincerely,

Doris T. Hollis

for Danny L. Laspe
Contract Specialist

Copy Furnished:
Laurence H. Baker, D.O.,
Principal Investigator

[illegible]

GRANT NO.	EFFECTIVE DATE	GRANT AMOUNT	PAGE 1 OF 1
DAMD17-94-J-4385 Modification P7000[illegible]	See Grants Officer Signature date below	$798,290.00	Danny L. Laspe (301) 619-7147

PROJECT TITLE:
Gene Therapy of H[illegible] [illegible] Cancer (AIBS #2450)

PERFORMANCE PERIO[illegible]	PRINCIPAL INVESTIGATOR:
1 October 1994 - 3[illegible] Oc[illegible] 1998 (Research ends 30 Sept[illegible] 1998)	Laurence H. Baker, D.O.
AWARDED AND ADMINISTERE[illegible] BY: U.S. ARMY MEDICAL SERVI[illegible] ACQUISITION ACTIVITY ATTN: MCMR-AAA- A FORT DETRICK FREDERICK, MARYLAND [illegible]02 5014	**PAYMENT WILL BE MADE BY:** DFAS-ROME (800) 553-0527 ATTN: VENDOR PAY, RO-FVP 134 CHAPPIE JAMES BOULEVARD ROME, NY 13441-4511
AWARDED TO: Regents of the Uni[illegible] [illegible] Michigan 3003 S. State Stre[illegible] P.O. Box 520 Ann Arbor, Michigan 4[illegible] [illegible]74	**REMIT PAYMENT TO:** Regents of the University of Michigan 3003 S. State Street P.O. Box 520 Ann Arbor, Michigan 48109-1274

ACCOUNTING AND APP[illegible]PRIATION DATA:
NO CHANGE

SCOPE OF WORK:

GRANT SCHEDULE

A. The Principal Invest[illegible] is changed from John Smith II, M. D., to Laurence H. Baker, D.O.

TOTAL AMOUNT OF AWARD: $ [illegible] (see Payment Section for Disbursement Schedule)

RECIP[illegible]	GRANTS OFFICER
ACCEPTED BY: SIG[illegible]TURE	**UNITED STATES OF AMERICA.** BY: Jean M. Shinbur SIGNATURE
NAME AND TITLE: No Signature Requi[illegible]ed Grantee's Letter D[illegible]ed [illegible] 1996.	NAME AND TITLE: JEAN M. SHINBUR GRANTS OFFICER
DATE	DATE: 7 Feb '97

GRANDAA FORM 25-R-E, APR [illegible] (Previous editions are obsolete)

DIVISION OF RESEARCH DEVELOPMENT AND ADMINISTRATION

PROJECT AWARD CHANGE

NO. 1 **Date: 3/4/97**

Rift 6287

To: Laurence H. Baker
Internal Medicine

From: Neil [illegible] (763-6438), Project Representative

RE: Change of project director

Project Title: Gene therapy of Human Breast Cancer

Sponsor: U S Army Medical

Award Reference: DAMD 94 J 4385

Modification no: P7000

Account Number: 0[illegible]226[illegible] **DRDA Number:** 941453

PLEASE NOTE THE FOLLOWING MODIFICATION TO YOUR PROJECT ACCOUNT:

The Principal Investigator is changed from John Smith II, M.D., to Laurence H. Baker, D.O.

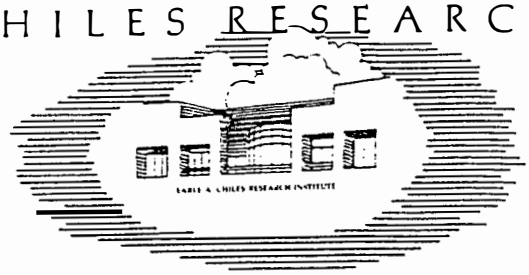
Post-it® Fax Note 7671	Date 4/7/97	# of pages ▶ 3
To Mary / Pam Stout	From D. Stanislaw	
Co./Dept.	Co. DRDA	
Phone #	Phone # 763-6438	
Fax # 6-7376	Fax #	

Please feel free to call your USC DRDA Project Representative to assist you in dealing with any administrative problems you may encounter in carrying out this project. Business matters should be addressed to Financial Operations. The person in Financial Operations responsible for your account is William Barnett 763-7971

cc: I. J. Goldstein
T. Yamada
Amy Laura
P. Stemple: Please execute and return [illegible] copy(ies) to the sponsor

ndg

EARLE A. CHILES RESEARCH INSTITUTE



Appendix 3

February 25, 1997

Rita Chatman
University of Michigan
1500 E. Medical Center Drive
Box 0374
Ann Arbor, MI 48109

RE: DOD Grant "Gene Therapy of Human Breast Cancer" DAMD17-94-J-4385, DRDA 941453: Subcontractor Agreement for John W. Smith II, M.D.

Dear Rita:

Enclosed are the documents required to establish the subcontractor agreement on the above-stated grant:

1. Biosketches for Key Personnel
   a. John W. Smith II, M.D.
   b. Deric D. Schoof, Ph.D.
2. Other Support for Key Personnel
   a. John W. Smith II, M.D.
   b. Deric D. Schoof, Ph.D.
3. Letter from Dr. Smith indicating reason for collaboration
4. Facility Description
5. Environmental Compliance
6. Safety Compliance
7. List of members of Providence Health System Institutional Review Board. As discussed, the clinical trial will be approved by our IRB when appropriate.

Please contact me should you require additional information (tel: 503-215-6580). Thank you again for your assistance in this project.

Sincerely,

Mary Kalez
Research Administrator

PROVIDENCE PORTLAND MEDICAL CENTER 4805 N.E. GLISAN STREET, PORTLAND, OR 97213-2967 PHONE (503) 215-6588

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
John W. Smith II	Associate Professor

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Amherst College, Amherst, MA		1973	
University of Pennsylvania, Philadelphia, PA	B.A.	1976	
Jefferson Medical College, Philadelphia, PA	M.D.	1981	Medicine

RESEARCH AND/OR PROFESSIONAL EXPERIENCE:

Professional Experience:

1986-1990	Senior Staff Fellow, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland
1990-1993	Medical Officer, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland
1993-1996	Associate Professor, University of Michigan Hospitals, Department of Internal Medicine, Division of Hematology/Oncology
1996-	Chief, Clinical Research, EACRI, PPMC, Portland, OR
1996-	Assistant Medical Director, Regional Cancer Program, Providence Health Systems, Portland, OR

Honors and Awards:

1971	National Merit Scholarship Qualifying Exam - Letter of Commendation
1976	B.A., Magna Cum Laude, with distinction in major, University of Pennsylvania
1980	Who's Who Among American Universities and Colleges, Jefferson Medical College
1993	National Institute of Health Merit Award, "For the development of the first cytokine therapy capable of significantly accelerating platelet recovery after high-dose chemotherapy", October 20, 1993.

Bibliography (selected from 38 peer reviewed publications):

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2. Steis, R.G., Urba, W.J., VanderMolen, L.A., Bookman, M.A., Smith II, J.W., Clark, J.W., Miller, R.L., Crum, E.D., Beckner, S.K., McKnight, J.E., Ozols, R.F., Stevenson, H.C., Young, R.C., and Longo D.L.: Intraperitoneal lymphokine-activated killer-cell and interleukin-2 therapy for malignancies limited to the peritoneal cavity. J. Clin. Oncol 8: 1618-1629, 1990.
3. Steis, R.G., Smith II, J.W., Urba, W.J., Venzon, D.J., Longo, D.L., Barney, R. Evans, L.M., Itri, L.M., and Ewel, C.H. Loss of interferon antibodies during prolonged continuous interferon-α2a therapy in hairy cell leukemia. Blood 77: 792-798, 1991.
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of intraperitoneal Pseudomonas exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer. J. Clin. Oncol.9:2095-2103, 1991.

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21. Johnson, T.M., Smith, J.W., Nelson, B.R., and Chang, A. Current Therapy for Cutaneous Melanoma. J Am Acad of Dermatol (In press, May 1995).
22. Curti, B.D. and Smith, J.W. Interleukin-1 in the Treatment of Cancer. Pharmac. Ther. 65:291-302, 1995.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Deric D. Schoof, Ph.D.	Chief, Division of Cellular Immunology

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
California State University, Sacramento	B.S.	1976	Biology
University of California, Berkeley	Ph.D.	1984	Immunology
University of California, San Francisco	Fellow	1984	Cellular Imm.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. K personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Experience:

1986-88 Instructor in Surgery, Harvard Medical School,Boston, MA
1989-93 Assistant Professor of Surgery, Harvard Medical School, Boston, MA
1993-present Associate Member, E.A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR
1993-present Member, Oregon Cancer Center, Oregon Health Sciences University, Portland, OR
1994-present Associate Professor of Medicine, Oregon Health Sciences University, Portland, OR
1994-present Associate Professor of Molecular Microbiology and Immunology Oregon Health Sciences University, Portland, OR

Hospital Appointments:

1986-93 Director, Laboratory of Biologic Cancer Therapy, Brigham & Women's Hospital, Boston, MA
1993-present Chief, Division of Cellular Immunology, E. A. Chiles Research Institute, Providence Medical Center, Portland, OR

Honors and Awards:

1980 University of California Regents Fellowship
1982 Grossman Endowment for dissertation research proposal
1983 Chancellor's Patent Fund for dissertation research proposal
1984 Brian Rydpath Award

Bibliography:

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5. **Eberlein, T. J., D. D. Schoof, S. E. Jung, D. Davidson, B. Gramolini, K. McGrath, A. Massaro, and R. E. Wilson.** 1988. A new regimen of interleukin 2 and lymphokine-activated killer cells. Efficacy without significant toxicity. *Archives of Internal Medicine 148:2571.*
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8. **Schoof, D. D., and C. H. Tempelis.** 1986. The role of soluble protein A in chicken spleen cell activation. *Developmental & Comparative Immunology 10:247.*
9. **Schoof, D. D., P. Hunt, J. A. Obando, J. Cusack, Jr., V. Andrews, Y. Terashima, and T. J. Eberlein.** 1992. Secondary cytokine production by lymphoid cells used in cellular immunotherapy. *Surgical Oncology 1:163.*
10. **Schoof, D. D., A. F. Massaro, J. A. Obando, J. Kusack, Jr., and T. J. Eberlein.** 1992. The biological effects of immunosuppression on cellular immunotherapy. *Surgical Oncology 1:27.*
11. **Peoples, G. E., P. S. Goedegebuure, J. V. Andrews, D. D. Schoof, and T. J. Eberlein.** 1993. HLA-A2 presents shared tumor-associated antigens derived from endogenous proteins in ovarian cancer. *J. Immunol. 151:5481.*
12. **Peoples, G. E., M. P. Davey, P. S. Goedegebuure, D. D. Schoof, and T. J. Eberlein.** 1993. T cell receptor V beta 2 and V beta 6 mediate tumor-specific cytotoxicity by tumor-infiltrating lymphocytes in ovarian cancer. *J. Immunol. 151:5472.*
13. **Schoof, D. D., J. A. Obando, J. Cusack, Jr., P. S. Goedegebuure, H. Brem, and T. J. Eberlein.** 1993. The influence of angiogenesis inhibitor AGM-1470 on immune system status and tumor growth in vitro. *International Journal of Cancer 55:630.*
14. **Schoof, D. D., Y. Terashima, G. E. Peoples, P. S. Goedegebuure, J. V. Andrews, J. P. Richie, and T. J. Eberlein.** 1993. CD4+ T cell clones isolated from human renal cell carcinoma possess the functional characteristics of Th2 helper cells. *Cell. Immunol. 150:114.*
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20. **Massaro, A. F., D. D. Schoof, A. Rubinstein, M. Zuber, F. J. Leonard-Vidal, and T. J. Eberlein.** 1990. Solid-phase anti-CD3 antibody activation of murine tumor-infiltrating lymphocytes. *Can. Res. 50:2587.*
21. **Yoshino, I., D. D. Schoof, G. E. Peoples, and T. J. Eberlein.** 1993. The sensitivity of ovarian epithelial tumor cells to cytotoxic T cells is associated with the expression of her2/neu. *Surgical Forum 44:492.*

22. **Schoof, D. D., W. J. Urba, G. Hughes, A. Rosenbloom, A. Yeo, A. Jones, and A. D. Weinberg.** 1995. Polarized cytokine gene expression by CD4+ helper T cells that infiltrate human renal cell carcinoma. *Submitted.*
23. **Schoof, D. D., C. M. Selleck, A. F. Massaro, S. E. Jung, and T. J. Eberlein.** 1990. Activation of human tumor-infiltrating lymphocytes by monoclonal antibodies directed to the CD3 complex. *Can. Res. 50:1138.*
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27. **Bertagnolli, M. M., S. H. Herrmann, V. M. Pinto, D. D. Schoof, and T. J. Eberlein.** 1991. Approaches to immunotherapy of cancer: characterization of lymphokines as second signals for cytotoxic T-cell generation. *Surgery 110:459.*
28. **Schoof, D. D., A. F. Massaro, J. A. Obando, J. C. Kusack, Jr., and T. J. Eberlein.** 1992. The biological effects of immunosuppression on cellular immunotherapy. *Surg Oncol 1:27.*
29. **Schoof, D. D., P. Hunt, J. A. Obando, J. C. Cusack, Jr., V. Andrews, Y. Terashima, and T. J. Eberlein.** 1992. Secondary cytokine production by lymphoid cells used in cellular immunotherapy. *Surg Oncol 1:163.*
30. **Schoof, D. D., Y. Terashima, S. Batter, L. Douville, J. P. Richie, and T. J. Eberlein.** 1993. Survival characteristics of metastatic renal cell carcinoma patients treated with lymphokine-activated killer cells plus interleukin-2. *Urology 41:534.*
31. **Goedegebuure, P. S., L. M. Douville, H. Li, G. C. Richmond, D. D. Schoof, M. Scavone, and T. J. Eberlein.** 1995. Adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 in patients with metastatic malignant melanoma and renal cell carcinoma: a pilot study. *J Clin Oncol 13:1939.*
32. **Mendez, M. V., R. G. Molloy, O. R. DS, R. G. Holzheimer, M. S. Nestor, A. Obando, I. Saporoschetz, D. D. Schoof, J. A. Mannick, and M. L. Rodrick.** 1993. Lymphokine activated killer cells enhance IL-2 prevention of sepsis-related death in a murine model of thermal injury. *J Surg Res 54:565.*

OTHER SUPPORT: JOHN W. SMITH II, M.D.

ACTIVE

1. **Role**: Principal Investigator **Dates of Project**: to begin Spring 1997 **Effort**: 0%
   Tot. Level of Funding: $90,000
   Supporting Agency: Immunex
   Title of Project: Phase I Study of Escalating Doses of GM-CSF Combined with a Fixed Low-Dose IL-2 Regimen
   Overlap: None

2. **Role**: Principal Investigator **Dates of Project**: 11/96 - 10/99 **Effort**: 5%
   Tot. Level of Funding: $50,000
   Supporting Agency: Genetics Institute, Inc.
   Title of Project: Phase III Double-Masked, Randomized Study of Recombinant Human Interleukin-11 at a Dose of 50 ug/kg Subcutaneously Once Daily for 14 days vs. Placebo in Adult Cancer Patients with Severe Thrombocytopenia Due to Chemotherapy
   Overlap: None

OTHER SUPPORT: DERIC D. SCHOOF, PH.D.

ACTIVE

1. **Project Number**: R03-CA70299-01 **Dates of Project**: 09/15/96 - 08/31/98 **Effort**: 20%
   Principal Investigator: Walter J. Urba, M.D., Ph.D. **Annual Direct Costs**: $49,998
   Source: NIH
   Title of Project: Vaccination with B7-transfected Breast Cancer Cells

   The major **goals** of this project are: (1) To measure the toxicity following vaccination with the B7 (CD80)-transfected, allogeneic breast cancer cell line, MDA-MB-231 and to observe patients for tumor regression; (2) To assess the immunologic response to vaccination of lymphocytes isolated from lymph nodes draining the vaccination site following a single dose of the B7-transfected allogeneic cell line, MDA-MB-231; (3) To assess the development of systemic immunity following multiple vaccinations with the B7-transfected allogeneic cell line, MDA-MB-231.

2. **Project Number**: NA **Dates of Project**: 09/01/93 - 09/01/97 **Effort**: 40%
   Principal Investigator:Deric D. Schoof, Ph.D. **Annual Direct Costs**: $100,000
   Source: Sister of Providence Health System in Oregon
   Title of Project: The immune response to renal cell cancer

   The major **goals** of this project are: (1) To determine the functional subsets of T cells infiltrating renal cell carcinoma; (2) To determine if T cell receptor signaling is impaired; (3) To determine if tumor suppresses T cell function.

PENDING

None

OVERLAP

None

John W. Smith II, M.D.
Chief, Clinical Research
Earle A. Chiles Research Institute
Providence Portland Medical Center
4805 NE Glisan Street, Suite 5F40
Portland, OR 97213-2967

October 8, 1996

Pam Stout, Division Administrator,
Rita Chatman, Grants Administrator
University of Michigan
Department of Internal Medicine
Division of Hematology/Oncology
3119 Taubman Center /0374
1500 E. Medical Center Drive
Ann Arbor, MI 48109

RE: DOD Grant "Gene Therapy of Human Breast Cancer", DAMD17-94-J-4385, DRDA 941453

Dear Pam and Rita,

Now that the dust is finally settling after my move, I am writing to you to furnish all the information necessary for you to submit a request for a subcontract on the above referenced grant to Kathryn R. Warner, Contracts Administrator as outlined in her letter that you gave me.

1. *Name of the subcontractor and the address of their contracts/grants office.*

3/1/97
Note change

~~Stephen G. Franey~~ Mary Kalez
~~Regional Director, Education & Research~~
Institutional Official (Acting)
Providence Portland Medical Center
4805 NE Glisan Street
Portland, OR 97213
(503) 215-6869

2. *Proposal from the subcontractor including the statement of work and the budget.*

The subcontractor, John W. Smith II, M.D., who served as the principal investigator for the first two years of the grant, proposes to serve as the Co-Principal Investigator for the last two years of the grant. The other Co-Principal Investigator will be Alfred Chang M.D. who served as an associate investigator the first two years of the grant. Dr. Smith will help coordinate the successful

performance of the four tasks described in detail in the statement of work on page 24 of the grant (attached). Task 3, conducting a Phase I clinical trial of B7 transfected breast cancer cells as a vaccine, and Task 4, performing immunologic monitoring studies on the patients participating in the trial, will occur both at the University of Michigan and at Providence Portland Medical Center. The joint enrollment of patients will be advantageous because the patient population (i.e. breast cancer patients with an easily obtainable source of autologous tumor) is relatively rare. Patient accrual will be improved by having both centers involved. Because the start of the clinical trial has been delayed by difficulty in manufacturing the adenoviral vector, there is less time to conduct the study and it would be extremely difficult for a single institution to complete the trial within the remaining time on the grant.

The Earle A. Chiles Research Institute, Portland Providence Medical Center was the first institution to conduct a gene therapy protocol in the state of Oregon. Currently, there are two active gene therapy studies at their institution, including a protocol for breast cancer patients, funded by the NIH, that is similar to the Army grant. Their proposal differs because it uses an HLA-A2 matched breast cancer cell line instead of autologous tumor, but both are transducing the breast cancer cells with B7 (CD80) to make the tumor immunogenic. Therefore, Dr. Smith and his colleagues at the The Earle A. Chiles Research Institute are completely capable and eminently qualified to conduct the clinical protocol and immunological monitoring as described in the statement of work.

Dr. Smith will be in frequent contact with his collaborators and in addition, will make two trips per year to the University of Michigan for on site meetings. The budget is attached.

3. *Period of performance for the subcontract.*

November 1, 1996 through October 31, 1998

4. *Subcontractor's key personnel.*

John W. Smith II, M.D., Principal Investigator
Deric D. Schoof, Ph.D., Co-Investigator
Teri Doran, R.N., Research Nurse
Judy Richardson, Research Assistant
Carolyn Kowalski, Data Manager

5. *Copy of the prime contract/grant and the University account number assigned to the project.*

You or Pam or Judy or all of you have a copy of the grant. The University of Michigan account number is 032266.

6. *Detailed explanation of why and how the proposed subcontractor was selected, including the number of bids obtained.*

The proposed subcontractor, John W. Smith II, M.D., wrote and submitted the grant proposal in November 1993 while he was at the University of Michigan in the Department of Internal Medicine, Division of Hematology/Oncology. The announcement that the grant was funded came the following spring and funding began in October 1994. Dr. Smith has served as the principal investigator for the first two years of the grant. In August, 1996, Dr. Smith left the University of Michigan to become the Chief of Clinical Research, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR. The DOD was contacted and they indicated that Dr. Smith could still be involved in the project through a subcontract made to his new institution. Dr. Laurence Baker, Associate Director of the University of Michigan Comprehensive Cancer Center, has indicated that he would like to keep Dr. Smith involved in the grant to insure that this important clinical research project be completed. Dr. Smith was selected as the proposed subcontractor because he has been coordinating this project as the principal investigator for the last two years. Because no other individual has the intimate knowledge of this grant that Dr. Smith has, no other bids were obtained.

7. *The State Conflict of Interest requires that a disclosure be made...*

There is no conflict of interest between Dr. Smith and the University of Michigan.

If there are any questions or items that need to be discussed, please contact me at 503-215-3635. Thank you in advance for your cooperation and assistance.

Sincerely,

John W. Smith II, M.D.

Subcontractor Agreement
Institution: University of Michigan
Subcontractor: The Earle A. Chiles Research Institute (P.I.: John W. Smith II, M.D.)

NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS 19.4%	TOTALS
John W. Smith II	Principal Investigator	12	10	$110,000	$11,000	$2,134	$13,134
Deric D. Schoof	Co-Investigator	12	5	$80,000	$4,000	$776	$4,776
Teri Doran	Research Nurse	12	5	$40,000	$2,000	$388	$2,388
Rachel Whitaker	Research Assistant	12	20	$17,680	$3,536	$686	$4,222
Carolyn Kowalski	Data Manager	12	10	$30,000	$3,000	$582	$3,582
TOTAL DIRECTS					**$23,536**	**$4,566**	**$28,102**
INDIRECTS @ 51%							**$42,434**
TOTAL BUDGET FOR 2 YEARS							**$84,868**

FACILITIES DESCRIPTION

No government-owned facilities or equipment are proposed for use with this project.

Providence Portland Medical Center (PPMC) is a not-for-profit tertiary medical center located in Portland, Oregon, with 483 licensed beds and 16,500 admissions per year. The hospital has 700 active medical staff members covering all medical and surgical subspecialties. It is owned and operated by the Sisters of Providence Health System whose corporate offices are located in Seattle, Washington.

PPMC has a strong commitment to cancer care, and has the largest number of new cases (approximately 1400 annually) of neoplastic disease diagnosed at a community hospital in the State of Oregon. Clinical capabilities include a full range of diagnostic and therapeutic services. Its 19-bed dedicated inpatient oncology unit is staffed by nine medical oncologists. An active 28,000 square foot radiation oncology department equipped with three state-of-the-art linear accelerators is staffed by three board-certified radiation oncologists. For the past seven years, PPMC has been the fiscal agent, and maintained central offices for an NCI-funded Community Clinical Oncology Program.

The Earle A. Chiles Research Institute (EACRI) has an investigative staff of 65, and an annual budget of approximately $4,000,000. Built in 1988, the EACRI serves as a center for both basic and applied research. The mission of the Institute is to improve patient care and enhance Providence's role as a major tertiary care referral center. The EACRI is formally affiliated with Oregon Health Sciences University for purposes of collaborative research. The EACRI occupies 21,000 square feet in Providence Portland Medical Center, adjacent to the inpatient oncology unit. It is presently comprised of 15,000 square feet of finished laboratory space, and 6000 square feet of unfinished space. Funds for completion are set aside for future development of the EACRI. The space is well-planned to provide for efficient sharing of common areas and equipment located along a central corridor. Shared areas include a cold room, six tissue culture rooms, utility room and conference facility.

The laboratories, which include the Molecular and Tumor Immunology Lab, the Cellular Immunology Lab, and the Basic Immunology Lab, occupy 3100 square feet in the west wing of the EACRI, and two separate 200 square foot closed labs in the east wing of the institute which are used exclusively to work with cells that will be given to patients and for recombinant DNA work. The labs are equipped with 6 six-foot biological safety cabinets, four two-chamber water-jacketed incubators and two large reach-in incubators (Forma Scientific), seven brightfield and six inverted microscopes (Zeiss), four refrigerated centrifuges and four microfuges. Radioisotopes are handled in a chemical hood designed specifically for this type of work. Radionuclides are counted on either a Packard Tri Carb 1900 TR liquid scintillation counter or a micromedic ME plus automatic gamma counter. There is also a MASH unit for harvesting labelled cell cultures. Samples of tissue, cell cultures, etc. are cryopreserved in two liquid nitrogen freezers (Cryomed). There is a common walk-in refrigerator for storing reagents and four -20° C freezer in the lab and four -80°C to -120°C freezers. Other equipment include two Elisa Readers, spectrophotometer. There is also access to three polymerase chain reaction and two flow cytometers.

The laboratory facility also contains separate office space for up to eight research fellows and technicians and is equipped with a computer work-station, modems and fax machines. The offices are also equipped with computer work-stations and have full secretarial support.

SAFETY PROGRAM PLAN

The Earle A. Chiles Research Institute at Providence Portland Medical Center has an existing safety plan program in accordance with appropriate Federal, state, and local regulations, as required by the Occupational Safety and Health Act. Hazards have been identified, eliminated and/or controlled and research may be performed safely under the laboratory conditions. Specific information on this plan is attached.

Additional assurances/certifications include:

- Human Subjects
- Vertebrate Animals
- Debarment and Suspension
- Drug-Free Workplace
- Lobbying
- Delinquent Federal Debt
- Research Misconduct
- Civil Rights
- Handicapped Individuals
- Sex Discrimination
- Age Discrimination
- Financial Conflict of Interest

DeAnn Wilson

11/13/96

DeAnn Wilson
Safety Officer
Providence Portland Medical Center

Date

Safety Program Plan

A. Research Operations- safety procedures used in the research operation

All laboratory personnel who will participate in this research program are required to take one full day of training sponsored by Providence Portland Medical Center which includes CPR, and 7 elements of the Environment of Care (safety) Program. Personnel receive instruction in a) general safety and ergonomics; b) infection control and protection against blood-borne pathogens; c) hazardous materials, the use of PPE and MSDS; d) emergency preparedness; e) fire safety; f) security g) utility management.

Safety Protocols for Standing Operating Procedures – The Institutional Biosafety Committee of the Earle A. Chiles Research Institute(EACRI)/Providence Portland Medical Center has established and implemented Guidelines deemed necessary to provide for the safe conduct of research and ensure compliance with its Guidelines for work performed in the EACRI. Laboratory personnel who will participate in this investigation receive explicit and detailed instruction on the discharge of their activities by the Co-Principal Investigator. Included in this is instruction of the Guidelines, their implementation and laboratory safety.

During the course of study proposed in our application, several different experimental protocols will be used. In terms of laboratory safety, however, the protocols can be grouped into three composite categories involving: 1) **cell culture** 2) the use of **radionuclides** and 3) **chemical exposure and toxic substances.**

Cell Culture. Laboratory protocols used in the proposed studies have been approved by the Co-Principal Investigator. Each standard operating procedure includes a description of the procedure, materials and methods, expected results and potential pitfalls. Each protocol includes a section that describes potential hazards (health and environment) and safety procedures including the use of PPE. To ensure safe research and operations for cell culture studies, lab personnel use universal precautions when handling tissues and blood samples from cancer patients. Personnel are required to wear lab coats and impervious gloves when handling cells or fluids. All materials, including liquids or solids, that come into contact with cell culture material are either autoclaved before disposal or are inactivated in bleach before disposal. Emergency procedures are in place for handling spills, minimizing creation of aerosols, and emergency devices (ie eye wash, sinks) are available in case of eye or skin contact. Emergency medical care is available if warranted.

All blood and tissue handled during the proposed studies will be manipulated in a biological safety cabinet using disposable plastics and mechanical pipetting devices. Routine cell culture procedures include: cell counting, dilution and cell transfer with mechanical pipettes, cell culture in flasks and plates. Sealed samples are also subjected to centrifugation. Cell sorting will also be performed in the biological safety cabinet. Cell

surface antigen expression will be determined by flow cytometry. This analysis will be performed at the bench with gloves and lab coat.

Radionuclides. Special training-- Lab personnel are required to take radiation safety classes at the Oregon Health Sciences University, Department of Radiation Safety (Portland, OR). Documentation of radiation safety class participation is maintained on file for each employee at the Earle A. Chiles Research Institute. Lab employees also receive instruction on how to order and receive radionuclides, how to use radionuclides, how to dispose of radionuclides and record keeping. In addition, a surveillance system has been implemented in which each employee is required to wear their own dosimeter during work with radionuclides. Dosimeter exposure levels are monitored on a monthly basis. On an annual basis the Radiation Safety Officer for the EACRI provides each employee with written notification summarizing their radiation exposure as a result of badge surveillance.

Chemical exposure and toxic substances. MSDS for each chemical used during the course of this investigation are kept on file in the laboratory. These data are available at all times. Lab personnel receive instruction on the handling of chemicals, their use, disposal and potential hazards. Volatile chemicals are used in a chemical fume hood.

Compliance.

The EACRI Institutional Biosafety Committee is recognized by the Institute to have the responsibility and authority to implement and enforce compliance with the provisions of the Guidelines. Recognition of this responsibility and authority establishes accountability for safe conduct of research at the local level.

B. Facility Equipment and Description

The Earle A. Chiles Research Institute comprises 15,000 sq. ft. of finished laboratory space. The Cellular Immunology lab occupies 1,500 sq. ft. and is furnished with one biological safety cabinet and CO_2 incubator. The safety cabinet is inspected annually. The incubator is monitored daily for CO_2 levels. The lab is also equipped with light microscopes, inverted microscopes, 3 centrifuges, 1 PCR thermocycler, speed fuge, speed vac, cell sorting facility, and Coulter Epics XL 4-color flow cytometer (pathology) and chemical fume hood. In addition, shared instruments include a FACScan, a gamma counter and beta counter, liquid nitrogen storage, ELISA plate reader, walk-in cold room and autoclave. Cells are irradiated with a linear accelerator in the Radiation Oncology Department, Providence Portland Medical Center. Other equipment includes 5 PC-type computers and 5 Macintosh computers. Included are Fax machines, laser printers and administrative and secretarial support staff.

Lab personnel are required to wear lab coats at all times. In addition, they are required to wear impervious gloves when handling cell cultures etc. Protective eye wear is also available for each lab worker. Other safety equipment available to lab personnel includes

showers, eye washes, fire extinguishers as well as a digital security system. This security system prevents the entry of unauthorized persons into the research area; entry into the Institute cannot occur without specific authorization.

C. Hazard Identification

Potential hazards associated with the proposed research study include:

a. Exposure to NaN_3

NaN_3 is a preservative added to monoclonal antibodies that will be used in this study.

Hazard Analysis: the maximum credible event (MCE) would include overexposure which may cause allergic reactions in susceptible individuals.

Recommendations: this hazard is avoided by use of personal protective devices (PPD) including lab coat, gloves and eye protection.

b. Reactivity of NaN_3 in acid conditions

Hazard Analysis: NaN_3 decomposes to hydrazoic acid following exposure to acid. Decomposition of azide to hydrazoic acid in metal sink traps has resulted explosions following discharge of acid in sinks.

Recommendations: this MCE can be minimized or eliminated by flushing the sinks with large volumes of water before and after azide waste is discharged into the sink. Further, flushing the pipes with water before discharging acid-containing substances into the sink would prevent the generation of hydrazoic acid.

c. Exposure to phenol & guanidinium thiocyanate

Phenol is a component of the reagent (RNA STAT-60, Friendswood, TX) used to isolate total cellular RNA.

Hazard Analysis: phenol is a poison and ingestion can be fatal.
Hazard Analysis: Guanidinium thiocyanate is also a component of RNA STAT-60 and can be an irritant to skin.

Recommendations: this reagent should be used in a chemical fume hood. Further, the use of lab coats, impervious gloves and eye protection are required when working with this reagent.

d. Exposure to chloroform

Chloroform is used in the isolation and purification of total cellular RNA.

Hazard Analysis: chloroform is classified by the EPA as carcinogenic.

Recommendations: exposure is minimized by the use of lab coats, gloves and is used in a chemical fume hood.

e. Exposure to polyacrylamide

Studies will be performed that require the use of PAGE gels.

Hazard Analysis: polyacrylamide is a neurotoxin that can be absorbed through unbroken skin.

Recommendations: lab coats, gloves and eye protection are required for the preparation and use of PAGE gels.

f. Exposure to ethidium bromide

Studies will be performed that require the visualization of PCR products on agarose gels.
Hazard Analysis: ethidium bromide is a nucleic acid intercalating agent. Exposure can cause frame shift mutations.

Recommendations: exposure is minimized by the use of lab coats, gloves and eye protection. In addition, a particle mask is required whenever powder is weighed.

g. Exposure to human blood and tissue

The studies conducted in this investigation will utilize blood and tissue harvested from breast cancer patients.

Hazard Analysis: the MCE would be the transfer of infectious disease from the blood/tissue sample to the laboratory worker.

Recommendations: Universal Precautions which are designed to minimize the risk of exposure to infectious blood-borne or tissue-borne microbial or viral agents will be used in this study. The use of PPD as detailed above, is required for all procedures utilizing blood/tissue.

h. Exposure to beta- or gamma-emitting radionuclides

Beta- or gamma-emitting radionuclides are used in the proposed studies to measure cell proliferation or cell-mediated cytotoxicity.
Hazard Analysis: Ingestion, absorption through skin/mucous membranes or inhalation of radionuclide-containing aerosols represent the MCE.

Recommendations: in addition to lab coats, impervious gloves and barriers, protective eye wear is recommended when handling ^{3}H-thymidine or ^{51}chromium. A Geiger-Mueller survey meter equipped with batteries is always available to monitor the work environment. Separate, labeled containers for ^{3}H-thymidine or ^{51}chromium are maintained for solid waste; Liquid waste (^{3}H-thymidine or ^{51}chromium) is disposed of by release to sewerage effluents in accordance with practices and limits established by the 'Oregon Rules For The Control Of Radiation'. In addition to our own surveillance, the laboratory and counting equipment is inspected on a monthly basis by an external party. A written report of the results of 'wipe' tests is sent to the radiation safety officer of the Earle A. Chiles Research Institute for review and if necessary, action.

D. Health Hazards

The work proposed in this application involves risk of exposure to infectious agents from cell culture (blood/tissue from cancer patients), radiation (^{3}H and ^{51}Cr) and chemical exposure and toxic substances (ie NaN_3). In each case, the level of exposure risk is no different than other routine BL2 level work. Exposures to all health and environment hazards are minimized by the enforced use of PPE, training and good laboratory practice.

E. Pollution Prevention and Toxic Substances

Potentially hazardous or environmentally unacceptable items have been identified above (C. Hazard Identification). Alternative materials have not been suggested because we recognize, in each case, the use of these items provides the best possible results in our hands. We have addressed the potential problems associated with exposure of personnel to each of the items identified under part C. The risk of exposure to the public during research and/or operations, including transportation, packing and shipping resulting from laboratory research is considered minimal.

F. Radioactive Materials

Radionuclides are used at the EACRI under the authority of an NRC Broad Scope C license. Radioactive materials used in this study will be ^{3}H-thymidine and ^{51}Chromium. Solid ^{3}H and ^{51}Cr waste is allowed to decay in storage in a secured facility. Liquid waste is disposed of by release into sanitary sewerage. Both materials are readily dispersible in water; the quantity of licensed radioactive material that is released into the sewer in one month divided by the average monthly volume of water released into the sewer (ie the monthly average concentration) does not exceed the limits of $1x10^{-2}$ µCi/ml for ^{3}H-and $5x10^{-3}$ µCi/ml for ^{51}Cr as established by 'Oregon Rules For The Control Of Radiation'.

G. Recombinant DNA

The human CD80 (B7) cDNA was isolated by reverse transcriptase/PCR cloning using RNA from the human Burkitt's lymphoma cell line Raji by Fenton and colleagues. The PCR product is 970 base pairs in length and its coding region sequence corresponds exactly to the published sequence for human CD80. This cDNA was cloned into the Xho 1 site of vector BCMGNeo, downstream of the cytomegalovirus early promoter/enhancer, to generate the plasmid BCMGNeo-B7. Sequences within BCMG-Neo encoding early region genes from the bovine papilloma virus (BPV 69%) were excised by digesting BCMGNeo-B7 with Hind III and Not I, and the large Hind III fragment resulting from this digestion was subcloned into the vector Bluescript SK-. The resulting plasmid CMV-B7 contains the CMV promoter driving expression of the human B7 gene and the SV40 promoter upstream of the neomycin phosphotransferase gene. No other eukaryotic genes are expressed. Other sequences include an intron and poly(A) site from the rabbit β–globin gene, and a filler fragment from the human β–globin gene. The colE1 origin, f1 origin and ampicillin resistance gene are derived from Bluescript SK-. All DNA segments present in this construct can be accounted for.

The proposed reset involves the administration of genetically-modified tumor cells to cancer patients. Genetic modification was performed by lipofecting a plasmid (pCMV-B7) into tumor cells (MDA-MB-231). The plasmid integrates into the host DNA and cannot exist as an episome and cannot replicate. Furthermore, retroviruses will not be used in this study nor will oncogenic viruses, toxin genes or co-cultivation procedures.

The IBC of the EACRI has approved the use of the transfected cell lines as proposed in this study finding that no unusual safety risk is posed to the EACRI/Providence Portland Medical Center or the surrounding community(Appendix XXX).

H. Other Safety Documentation

Not applicable.

APPENDIX 2

Certificate of Environmental Compliance

CERTIFICATE OF ENVIRONMENTAL COMPLIANCE

The offeror currently ___ IS ___ IS NOT (check appropriate category) in compliance with applicable national, state, and local environmental laws and regulations. (*If not in compliance, attach details and evidence of approved mitigation measures.*)

The offeror has examined the activities encompassed within the proposed action entitled " Gene Therapy of Human Breast Cancer"

_____" (*enter title and/or Solicitation number and Principal Investigator's name)*, for compliance with environmental laws and regulations. The offeror states that the conduct of the proposed action

1. WILL NOT violate any applicable national, state, or local environmental law or regulation.

2. WILL NOT have a significant impact on the environment.

The offeror agrees that if the work required under the proposed action at any time results in a significant impact on the environmental or violation of any applicable environmental law or regulation, the offeror will immediately take appropriate action, to include notifying and/or coordinating with the appropriate regulatory agencies as required by law and notifying the Contracting Officer.

DeAnn Wilson
Name of Official Responsible for Environmental Compliance

DeAnn Wilson
Signature

Safety Officer
Title

2-21-97
Date

Providence Portland Medical Center
Name of Organization

PROVIDENCE HEALTH SYSTEM
INSTITUTIONAL REVIEW BOARD
MEMBERSHIP LIST

ASSURANCE NUMBER: T-4078 (Expires 8/11/99)

Chairperson: Craig S. Fausel, M.D.
Address: Providence Portland Medical Center
5050 N.E. Hoyt, Plaza, Level B
Portland, OR 97213
Phone: (503) 215-6512

DATE: 2/25/97
REGIONAL SCOPE: Providence Portland Medical Center, Providence St. Vincent Medical Center, and Providence Milwaukie Hospital

MEMBER NAME FIRST, MI, LAST	PRIMARY SCIENTIFIC OR NONSCIENTIFIC SPECIALTY	AFFILIATION WITH INSTITUTION/ RELATIONSHIP TO PROVIDENCE HEALTH SYSTEM
Craig S. Fausel, M.D. Chairperson	Gastroenterology	PPMC - Medical Staff Member
Eric Friedman, M.D.	General Surgery	PPMC - Medical Staff Member
David Gilbert, M.D.	Internal Medicine; Infectious Diseases	PPMC - Medical Staff Member
Randall Gore, M.D.	Internal Medicine	PPMC - Medical Staff Member
Huldrick Kammer, M.D.	Internal Medicine; Endocrinology	PPMC - Medical Staff Member
Michael Mastanduno, M.D.	Medical Oncology	PMH - Medical Staff Member
Rebecca Orwoll, M.D.	Internal Medicine Hematology/Oncology	PPMC - Medical Staff Member

MEMBER NAME FIRST, MI, LAST	PRIMARY SCIENTIFIC OR NONSCIENTIFIC SPECIALTY	AFFILIATION WITH INSTITUTION/ RELATIONSHIP TO PROVIDENCE HEALTH SYSTEM
Marie Driever, R.N., Ph.D.	Assistant Director, Dept. of Nursing QA and Research	PPMC - Staff
Thompson Faller, Ph.D.	Professor of Philosophy; Ethicist (University of Portland)	Community Member
Joanne Jackson, R.N., M.S.	Infection Control Nurse Epidemiologist Quality Maintenance	PPMC - Staff
Sylvia McSkimming, R.N., Ph.D.	Nursing Administration Associate Director of Nursing Research and Education	PSVMC - Medical Staff Member
David Nikula, R.Ph., M.S.	Pharmacy	PPMC - Staff
Bob Peterson	President, Allied Power Products	Community Member
Joan Sifferle, M.B.A.	Medical Records Director, Information Services	PSVMC - Staff
Laurie Skokan, Ph.D.	Social Psychology, Health Psychology and Statistics Senior Research Associate - Cancer Center for Outcomes Research and Education (CORE)	Regional Staff

MEMBER NAME FIRST, MI, LAST	PRIMARY SCIENTIFIC OR NONSCIENTIFIC SPECIALTY	AFFILIATION WITH INSTITUTION/ RELATIONSHIP TO PROVIDENCE HEALTH SYSTEM
Madalyn Whitaker, B.S., R.Ph.	Director, Dept. of Pharmacy	PSVMC - Staff

EX OFFICIO MEMBERS:

Beth Getman, RN, BSN, OCN, Clinical Trial Nurse, CROP, SW Washington Medical Center Representative
William Olson, Providence Health System Administration/Financial Representative
Kathy Young, RN, BSN, OCN, Clinical Trial Nurse, CROP, Legacy Health Systems Representative

IRB STAFF:

Nancy Harp, Administrative Director
Jean Sork, RN, Research Study Coordinator
Pauline Waite, Administrative Secretary

MEMBERSH.LST

SUBCONTRACT BETWEEN
THE REGENTS OF THE UNIVERSITY OF MICHIGAN
AND
PROVIDENCE PORTLAND MEDICAL CENTER
ISSUED UNDER
DEPARTMENT OF THE ARMY GRANT NO. DAMD17-94-J-4385

This agreement is entered into as of the 1st day of November, 1996, by and between the Regents of the University of Michigan, a non-profit constitutional corporation, organized and existing under the laws of the State of Michigan, with offices in the city of Ann Arbor, Michigan (hereinafter referred to as "Michigan"), and Sisters of Providence in Oregon, dba Providence Portland Medical Center, organized and existing under the laws of the State of Oregon, with offices in the city of Portland, Oregon (hereinafter referred to as "Subcontractor").

Michigan is recipient of a Department of the Army, U.S. Army Medical Research Acquisition Activity Grant (hereinafter referred to as "prime agreement") for support of a project entitled "Gene Therapy of Human Breast Cancer", and Subcontractor agrees to participate in the project as set forth in this subcontract for the consideration stated herein.

The parties mutually agree to the following terms.

ARTICLE I - STATEMENT OF WORK

Subcontractor will use its best efforts to perform the work described in the statement of work attached as Exhibit A.

Subcontractor shall furnish all necessary labor, materials and facilities to carry out the specific objectives outlined in Exhibit A.

ARTICLE II - PERIOD OF PERFORMANCE

The period of performance for this subcontract shall be from November 1, 1996 through October 31, 1998, unless amended by written mutual agreement. Expenditures incurred prior to the beginning date or subsequent to the termination date are unallowable.

ARTICLE III - PAYMENT

The total cost of the work to be performed under this subcontract is $84,868. Michigan shall not be obligated to reimburse nor shall Subcontractor be obligated to incur any expenditures in excess of this limitation. The budget for this subcontract is attached as Exhibit B.

Subcontractor shall submit invoices **in duplicate** indicating the amount expended according to each budget category. The controller or some other responsible official shall certify on the invoice that the request for payment is true and correct to the best of their knowledge and that all expenditures reported have been made in accordance with the appropriate prime sponsor policies and for the purposes set forth in the subcontract. The invoices shall reference Purchase Order No. _____ and be mailed to:

The University of Michigan
Office of Contract Administration
2044 Wolverine Tower
3003 S. State Street
Ann Arbor, Michigan 48109-1273

The invoices shall be reviewed by the project director at Michigan and returned, with his approval, to the Office of Contract Administration for payment processing.

Upon request of Michigan, Subcontractor shall refund any amounts determined to be unallowable expenditures as a result of Federal or Michigan audit. Subcontractor however, shall have the right to establish allowability of any such item of cost under the subcontract.

The final billing shall be submitted no later than sixty (60) days after the end of the period of performance as indicated in Article II. In no event shall the final billing exceed the subcontract amount.

ARTICLE IV - GENERAL PROVISIONS

This subcontract is issued under U.S. Army Medical Research Acquisition Activity Grant No. DAMD17-94-J-4385 and is subject to the regulations attached hereto as Exhibit C.

All references to granting agency, government, etc., in the regulations cited above are understood to refer to Michigan; all references to grantee or recipient etc., are understood to refer to Subcontractor.

ARTICLE V - HOLD HARMLESS

To the extent allowable by state law, each party hereby assumes any and all risks of personal injury and property damage attributable to the negligent acts or omissions of that party and the officers, employees and agents thereof.

ARTICLE VI - EQUIPMENT

Title to equipment purchased under this subcontract will reside with Subcontractor. Inventory and accountability of equipment will be in accordance with policies of the prime sponsor.

ARTICLE VII - DISPUTES

Except as otherwise provided for in this subcontract, any dispute not disposed of by mutual consent shall be decided by procedures jointly agreed upon by Michigan and Subcontractor.

ARTICLE VIII - TERMINATION

This subcontract may be terminated by either party with thirty days written notice to the other party. Upon receipt of a termination notice, Subcontractor will make no further commitments under this subcontract and will take all reasonable actions to cancel outstanding obligations. Subcontractor will furnish all necessary reports of research completed or in progress through the date of termination.

ARTICLE IX - KEY PERSONNEL

The following individuals will be responsible for all aspects of the proposed work to be performed by the subcontractor: John W. Smith, II, M.D., Deric D. Schoof, Ph.D.

Substitutions for these individuals or substantial reduction in any of the level of effort will not be made without the prior written approval of Michigan.

ARTICLE X - PUBLICATIONS

Subcontractor is free to publish reports or results of the research being performed under this subcontract. Thirty days prior to such publication, however, the Subcontractor will submit to Michigan's project director a copy of the proposed publication.

ARTICLE XI - PUBLICITY

Neither Michigan nor Subcontractor will use the name of the other either expressly or by implication, in any news, publicity release, or other fashion without the express written approval of the other.

ARTICLE XII - LIABILITY AND INSURANCE

The Subcontractor agrees that it shall procure and maintain insurance as required by law or regulation. The Subcontractor also agrees that it has entered into this subcontract and will discharge its obligations, duties and undertakings and work pursuant thereto, whether requiring professional judgment or otherwise, as an independent agent without imputing liability on the part of Michigan for the acts of the Subcontractor and its employees.

Article XIII - ASSURANCES

Subcontractor certifies that:

1) It is not delinquent on the repayment of any Federal debt.

2) It is presently not debarred, suspended, proposed for debarment, declared ineligible, nor voluntarily excluded from covered transactions by any Federal department or agency.

3) It is in compliance with the Drug-Free Workplace Act of 1988.

4) It is in compliance with Public Law 101-121, Section 1352 which covered restrictions regarding lobbying.

ARTICLE XIV - EQUAL OPPORTUNITY

During the performance of the work under this subcontract, Subcontractor shall not discriminate against any employee or applicant for employment because of race, color, religion, sex, national origin, handicap, special disability or special Vietnam era status. All the provisions of the non-discrimination clause set forth in Executive Order No. 11246, as amended, are hereby incorporated by reference into this subcontract.

ARTICLE XV - PATENTS AND INVENTIONS

Subcontractor agrees to comply with the Standard Patent Rights Clause of 37 CFR 401.14 or FAR 52.227 as appropriate.

ARTICLE XVI - REPORTS

Subcontractor shall prepare reports as requested by Michigan's project director. Only those reports will be requested that will enable Michigan to fulfill its commitments under its prime agreement.

ARTICLE XVII - AUDIT REPORTS

Subcontractor agrees to comply with the requirements of OMB Circular A-133 or Circular A-128 as appropriate. Subcontractor further agrees to provide Michigan with copies of any of the independent auditors' reports which present instances of non-compliance with federal laws and regulations which bear directly on the performance or administration of this subcontract. In cases of such non-compliance, subcontractor will provide copies of responses to auditors' reports and a plan for corrective action. All reports shall be prepared in accordance with the requirements of OMB Circular A-133 or A-128 as appropriate and sent to the following address:

University of Michigan
Office of Contract Administration
2044 Wolverine Tower
3003 S. State Street
Ann Arbor, Michigan 48109-1273

ARTICLE XVIII - PROJECT DIRECTOR

Michigan's project director is Laurence H. Baker. The project director is not authorized to amend or alter this subcontract. Any such amendments or alterations must be approved by the written mutual agreement of the parties hereto.

In witness whereof, the parties hereto have executed this subcontract as of the day and year first above written.

PROVIDENCE PORTLAND MEDICAL CENTER	THE REGENTS OF THE UNIVERSITY OF MICHIGAN
David T. Underriner	
Signature	Signature
David T. Underriner Operations Administrator	
Typed Name and Title	Typed Name and Title
4-17-97	
Date	Date

EXHIBIT A

The subcontractor, John W. Smith II, M.D., who served as the principal investigator for the first two years of the grant, proposes to serve as the Co-Investigator for the last two years of the grant. The other Co-Investigator will be Alfred Chang M.D. who served as an associate investigator the first two years of the grant. Dr. Smith will help coordinate the successful performance of the four tasks described in detail in the statement of work on page 24 of the grant (attached). Task 3, conducting a Phase I clinical trial of B7 transfected breast cancer cells as a vaccine, and Task 4, performing immunologic monitoring studies on the patients participating in the trial, will occur both at the University of Michigan and at Providence Portland Medical Center. The joint enrollment of patients will be advantageous because the patient population (i.e. breast cancer patients with an easily obtainable source of autologous tumor) is relatively rare. Patient accrual will be improved by having both centers involved. Because the start of the clinical trial has been delayed by difficulty in manufacturing the adenoviral vector, there is less time to conduct the study and it would be extremely difficult for a single institution to complete the trial within the remaining time on the grant.

The Earle A. Chiles Research Institute, Portland Providence Medical Center was the first institution to conduct a gene therapy protocol in the state of Oregon. Currently, there are two active gene therapy studies at their institution, including a protocol for breast cancer patients, funded by the NIH, that is similar to the Army grant. Their proposal differs because it uses an HLA-A2 matched breast cancer cell line instead of autologous tumor, but both are transducing the breast cancer cells with B7 (CD80) to make the tumor immunogenic. Therefore, Dr. Smith and his colleagues at the The Earle A. Chiles Research Institute are completely capable and eminently qualified to conduct the clinical protocol and immunological monitoring as described in the statement of work.

Dr. Smith will be in frequent contact with his collaborators and in addition, will make two trips per year to the University of Michigan for on site meetings. The budget is attached.

NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS 19.4%	TOTALS
John W. Smith II	Principal Investigator	12	10	$110,000	$11,000	$2,134	$13,134
Deric D. Schoof	Co-Investigator	12	5	$80,000	$4,000	$776	$4,776
Teri Doran	Research Nurse	12	5	$40,000	$2,000	$388	$2,388
Judy Richardson	Research Assistant	12	20	$17,680	$3,536	$686	$4,222
Carolyn Kowalski	Data Manager	12	10	$30,000	$3,000	$582	$3,582
TOTAL DIRECTS					**$23,536**	**$4,566**	**$28,102**
INDIRECTS @ 51%							**$42,434**
TOTAL BUDGET FOR 2 YEARS							**$84,868**

EXHIBIT B

EXHIBIT C

s contract incorporates one or more clauses by reference, with the same force and effect as if they were given in full text. n request, the Contracting Officer will make their full text available.

CLAUSE TITLE	REFERENCE	DATE
. ACQUISITIONS FROM SUBCONTRACTORS SUBJECT TO ON-SITE INSPECTION UNDER THE INTERMEDIATE - RANGE NUCLEAR FORCES (INF) TREATY	252.209-7000	(DEC 1991)
. COST ESTIMATING SYSTEM REQUIREMENTS	252.215-7002	(DEC 1991)
. PREFERENCE FOR DOMESTIC HAND OR MEASURING TOOLS	252.225-7015	(DEC 1991)
. RIGHTS IN TECHNICAL DATA AND COMPUTER SOFTWARE (ALTERNATE II (OCT 1988))	252.227-7013	(OCT 1988)
. RESTRICTIVE MARKINGS ON TECHNICAL DATA	252.227-7018	(OCT 1988)
. IDENTIFICATION OF TECHNICAL DATA	252.227-7029	(APR 1988)
. TECHNICAL DATA—WITHHOLDING OF PAYMENT	252.227-7030	(OCT 1988)
. DATA REQUIREMENTS	252.227-7031	(OCT 1988)
. PATENTS—SUBCONTRACTS	252.227-7034	(APR 1984)
. CERTIFICATION OF CLAIMS AND REQUESTS FOR ADJUSTMENT OR RELIEF	252.233-7000	(DEC 1991)
. RECOVERY OF MANUFACTURING COSTS AND ROYALTY FEES ON COMMERCIAL SALES	252.270-7000	(DEC 1991)
. DEFINITIONS	52.202-1	(SEP 1991)
. OFFICIALS NOT TO BENEFIT	52.203-1	(APR 1984)
. GRATUITIES	52.203-3	(APR 1984)
. COVENANT AGAINST CONTINGENT FEES	52.203-5	(APR 1984)
. ANTI-KICKBACK PROCEDURES	52.203-7	(OCT 1988)
. LIMITATION ON PAYMENTS TO INFLUENCE CERTAIN FEDERAL TRANSACTIONS	52.203-12	(JAN 1990)
NEW MATERIAL	52.210-5	(APR 1984)
USED OR RECONDITIONED MATERIAL, RESIDUAL INVENTORY, AND FORMER GOVERNMENT SURPLUS PROPERTY	52.210-7	(APR 1984)
EXAMINATION OF RECORDS BY COMPTROLLER GENERAL	52.215-1	(APR 1984)
AUDIT—NEGOTIATION	52.215-2	(DEC 1989)
PRICE REDUCTION FOR DEFECTIVE COST OR PRICING DATA	52.215-22	(JAN 1991)
PRICE REDUCTION FOR DEFECTIVE COST OR PRICING DATA—MODIFICATIONS	52.215-23	(DEC 1991)
SUBCONTRACTOR COST OR PRICING DATA	52.215-24	(DEC 1991)
SUBCONTRACTOR COST OR PRICING DATA — MODIFICATIONS	52.215-25	(DEC 1991)
ALLOWABLE COST AND PAYMENT note: Delete from paragraph (a) the words SUBPART 31.2 and substitute SUBPART 31.3	52.216-7	(JUL 1991)
COST CONTRACT—NO FEE (ALTERNATE I (1984 APR))	52.216-11	(APR 1984)
UTILIZATION OF WOMEN-OWNED SMALL BUSINESSES	52.219-13	(AUG 1986)
UTILIZATION OF LABOR SURPLUS AREA CONCERNS	52.220-3	(APR 1984)
LABOR SURPLUS AREA SUBCONTRACTING PROGRAM	52.220-4	(APR 1984)
NOTICE TO THE GOVERNMENT OF LABOR DISPUTES	52.222-1	(APR 1984)
WALSH-HEALEY PUBLIC CONTRACTS ACT	52.222-20	(APR 1984)
EQUAL OPPORTUNITY	52.222-26	(APR 1984)

EQUAL OPPORTUNITY PREAWARD CLEARANCE OF SUBCONTRACTS .....52.222-28..... (APR 1984)
AFFIRMATIVE ACTION FOR SPECIAL DISABLED AND VIETNAM ERA
VETERANS .....52.222-35..... (APR 1984)
AFFIRMATIVE ACTION FOR HANDICAPPED WORKERS .....52.222-36..... (APR 1984)
EMPLOYMENT REPORTS ON SPECIAL DISABLED VETERANS AND
VETERANS OF THE VIETNAM ERA .....52.222-37..... (JAN 1988)
CLEAN AIR AND WATER .....52.223-2..... (APR 1984)
RESTRICTIONS ON CERTAIN FOREIGN PURCHASES .....52.225-11..... (APR 1991)
UTILIZATION OF INDIAN ORGANIZATIONS AND INDIAN-OWNED
ECONOMIC ENTERPRISES .....52.226-1..... (AUG 1991)
AUTHORIZATION AND CONSENT (ALTERNATE I (APR 1984)) .....52.227-1..... (APR 1984)
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Immune Responsiveness to a Murine Mammary Carcinoma Modified to Express B7-1, IL-12 or GM-CSF

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Abstract

This report characterizes the immunological host response to a syngeneic murine mammary carcinoma along with variants genetically modified to express B7-1 or secrete GM-CSF and IL-12. The MT-901 is a subline of a mammary adenocarcinoma which was chemically induced in the Balb/c host. It was found to be weakly immunogenic by immunization/challenge experiments; and, induced tumor-specific T cell responses in lymph nodes (LN) draining progressive subcutaneous tumors. Tumor clones expressing B7-1 or secreting GM-CSF exhibited reduced tumorigenicity without completely abrogating tumor growth whereas IL-12 elaboration lead to complete tumor growth inhibition. In vivo s.c. inoculation of a transgenic cell clone secreting GM-CSF (240 ng/10^6 cells/24h) resulted in significantly enhanced T cell reactivity of tumor-draining LN (TDLN) cells as compared to wild-type TDLN cells. This finding was obtained from observations assessed by several different methods, including: 1) in vitro cytotoxicity, 2) in vitro IFNγ release, and 3) adoptive transfer in mice with established tumor. Moreover, the transfer of activated LN cells derived from mice inoculated with GM-CSF secreting tumor cells resulted in the prolonged survival of animals with macroscopic metastatic disease which was not evident utilizing LN cells from mice inoculated with wild-type tumor. By contrast, clones that expressed B7-1 or IL-12 (4 ng/10^6 cells/24h) did not elicit enhanced tumor reactive TDLN cells compared with wild-type tumor when assessed in the adoptive transfer model. The autocrine secretion of GM-CSF by transduced tumor cells was found to serve as an effective immune adjuvant in the host response to this weakly immunogenic tumor.

Introduction

Recently, there has been increased interest in the development of genetically engineered vaccines for the treatment of malignancy. Several reports have evaluated a variety of immunoregulatory molecules expressed by transfected tumor cells which manifest enhanced immunogenicity when inoculated into syngeneic hosts (1-3). In general, this enhanced immunogenicity is observed as decreased tumorigenicity mediated by host immune cells induced to be reactive against tumor-associated antigens during initial tumor growth. In select instances, the administration of genetically modified tumor cells as a vaccine has resulted in the rejection of established wild-type tumors (4,5).

Due to the limitations of vaccines in the therapy of significant tumor burdens, we have focused our efforts at employing vaccines to elicit tumor reactive T cells which can be further expanded ex vivo for subsequent adoptive immunotherapy. The adoptive transfer of tumor reactive T cells in experimental models has been shown to result in the significant regression of localized as well as metastatic tumors; and to provide long-lasting immunity (6). We have developed a method to retrieve and expand immune T cells from vaccine-primed lymph nodes (LN) which involves the sequential culturing of lymphoid cells with anti-CD3 monoclonal antibody (anti-CD3) followed by exposure to IL-2 (7-10).

In this report, we have characterized the immunogenicity of a murine mammary carcinoma which has not been previously described. Furthermore, we evaluated the immunogenicity of this tumor after transfection with three different immunomodulatory molecules which have been reported to upregulate immune responsiveness; namely GM-CSF, IL-12, and the co-stimulatory molecule B7-1 (11-13). We have used our adoptive transfer model for the treatment of established pulmonary metastases as the primary endpoint to assess T cell reactivity of lymphoid cells obtained from hosts inoculated with the transfected tumor cells. We

found that the local elaboration of transgenic GM-CSF consistently enhanced T cell sensitization to an inoculum of tumor cells compared with wild-type tumor cells; and was not evident with the tumor transfectants expressing B7-1 or IL-12. This finding extends our previous observations regarding the use of GM-CSF as an adjuvant for the induction of T cells reactive to a poorly immunogenic melanoma (14).

Materials and Method

Mice

Female Balb/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME. They were maintained in specific pathogen-free conditions and were used for experiments at age of 8 weeks or older.

Tumors

MT-7 is a cultured tumor cell line derived from a dimethylbenzanthracene (DMB)-induced mammary carcinoma in the Balb/c host provided by Dr. Robert L. Ullrich, University of Texas Medical Branch, Galveston, TX(15). The tumor was induced by intragastric lavage of DMB with subsequent appearance of tumor in mammary tissue. A subline, MT-901, was derived from an early in vivo passage of cultured MT-7 tumor inoculated subcutaneously (s.c.). Cells derived from this progressive tumor were cultured in complete media (CM) which consisted of RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Grand Island Biological Company, Grand Island, NY), and 5×10^5 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). Renca, a renal cell carcinoma syngeneic to the Balb/c mouse, was kindly supplied by Dr. Gary Nabel, University of Michigan, Ann Arbor, MI and used as a specificity control.

Genetic modification of cells

GM-CSF: MT-901 cells split 1:10 were used the next day for transduction with 5 ml of retrovirus supernatant from Ψ-crip cells transfected with an MFG-GM-CSF vector made in our laboratory (14) in the presence of polybrene (10 μl/ml). Bulk culture of transduced cells were cloned by the limiting dilution method and screened for cytokine production. GM-CSF transduced clones were isolated and measured to secrete 240 (MT-9G1) or 35 (MT-9G2) ng/10^6 cells/24h by ELISA.

B7-1: Tumor cells were transfected utilizing cationic polymers termed dendrimers. These reagents were kindly provided by Dr. James R. Baker, University of Michigan, Ann Arbor, MI. Briefly, DNA/dendrimer solution was made by combining 1 μg DNA with 2 μl of 10X DNA/dendrimer binding buffer (filtered 40% glycerol, 10 mM, 50 mM DTT and 100 mM NaCl), 15 μl filtered water, 13 μg G10EDA (Dendrimer) in 2 μl of dendrimer dilution buffer (20% glycerol, 20 mM pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT). Murine B7-1 plasmid was cloned into a pMV6-neo backbone. Upon mixing the DNA and dendrimer solution, it was allowed to stand for 15 min. at room temperature. Five x 10^6 MT-901 cells were washed 2 times with the Hanks' Balanced Salt Solution (HBSS, GIBCO) and incubated with 20 μl DNA/dendrimer solution for 2 hrs at 37°C. After 48 hr in culture with CM in a 10 cm petri dish, the media was exchanged and 0.6 mg/ml Geneticin (GIBCO) was added. Visible colonies were plucked and cultured. One of these clones was continuously cultured and found to stably express B7-1 over several weeks and termed MT-9B14.

IL-12: MT-901 was transfected with the IL-12 gene by standard electroporation methods. Briefly, 10^7 MT-901 cells in 0.5 ml RPMI-1640 was electroporated with 10 μg murine IL-12 and 1 μg of the neomycin resistance gene. The murine IL-12 cDNA expression plasmid in tandem construction was obtained as previously described (16). The cells were placed in CM and 48 hr later, 0.6 mg/ml of Geneticin was added. Visible colonies were plucked and cultured. MT-901.IL-12-4 was the highest expressing clone and found to secrete 4 ng/10^6 cells/24h by ELISA.

Tumorigenicity assessment

Mice were inoculated s.c. with the indicated number of tumor cells in 0.05 ml of HBSS. Tumors were measured twice per week in two perpendicular dimensions with a vernier caliper and the size recorded as an area (mm^2).

Tumor-draining LN cells and activation

Wild-type or modified tumor (1.5×10^6) in 0.05 ml HBSS was inoculated s.c. in the flank of syngeneic mice. Nine days later, tumor-draining lymph nodes (TDLN) were removed aseptically. Lymphoid cell suspensions were prepared by mechanical dissociation with 25-gauge needles and pressed with the blunt end of a 10 ml plastic syringe in RPMI-1640. The cells were activated with 1 μg/ml of anti-CD3 monoclonal antibody immobilized in 24-well plates (4×10^6 cells/2 ml/well) for 2 days as previously described (8). Anti-CD3 mAb was produced from 145-2C11 hybridoma cells which were a kind gift from Dr. Jeffrey A. Bluestone, University of Chicago, Chicago, IL (17). The LN cells were subsequently cultured in 60 IU/ml IL-2 for 3 days at 2×10^5 cells/ml. Human recombinant IL-2 was a gift from Chiron Therapeutics, Emeryville, CA. It had a specific activity of 18×10^6 international units/mg protein. On average, there was a 5- to 6-fold expansion of cells with this activation procedure.

Adoptive immunotherapy

Balb/c mice were inoculated i.v. with 3 to 5×10^5 MT-901 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were infused i.v. with activated TDLN cells and given i.p. injections of IL-2 (30,000 IU) in 0.5 ml of HBSS commencing on the day of cell transfer and continuing twice daily for 8 doses. On days 14 to 18, all mice were randomized and sacrificed for enumeration of pulmonary metastatic nodules. The metastases appeared as discrete white nodules on the black surface of lungs insufflated with a 15% solution of India ink and bleached by Fekete's solution (18). Metastatic foci too numerous to count were assigned an arbitrary value of >250. The significance of differences in numbers of metastatic nodules between experimental groups was determined using the non-parametric Wilcoxon rank-sum test. Two-sided p-values of <0.05 were considered significant. Each group consisted of a least 5 mice, and no animal was excluded from the statistical evaluation.

Immunofluorescence and flow cytometry

Tumor cells were analyzed for major histocompatibility class I and II molecules as well as B7-1 by direct immunofluoresence assay. Fluorescence isothiocyanate (FITC)-labeled mAb to H-2K^d (MHC Class I), and H-2I-A^d (MHC class II) from PharMingen, San Diego, CA were use for staining. Stained cell preparations were analyzed in a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA). Fluorescence profiles were generated by analyzing 10,000 cells and displayed as logarithmically increasing fluorescence intensity versus cell numbers.

Measurement of in vitro cytokine release by LN cells

One x 10^6 activated TDLN cells were co-cultured with 5 x 10^5 wild-type MT-901 tumor cells irradiated to 15,000 cGy in 2 ml of CM per well of a 24-well tissue culture plates. Sixty IU/ml of IL-2 was added at the beginning of the cultures which lasted for 48 hr at 37°C. The supernatants were collected, and after centrifugation, analyzed for IFNγ and GM-CSF measurements in duplicates using commercially available enzyme-linked immunoabsorbent assays (ELISA) obtained from PharMingen. Murine IL-12 ELISA reagents were a kind gift from Dr. David H. Persky, Hoffman-La Roche, Nutley, NJ. Both the p75 and p40 molecules of the IL-12 heterodimer are detected by this assay. If detectable, background cytokine values produced by tumor cells alone were subtracted from the co-culture values in the reporting results. For murine GM-CSF, a standard curve starting at 20 ng/ml with 11 serial 2-fold dilutions was performed. For IFNγ, a standard curve starting at 1,000 u/ml was established in a similar fashion. Experimental values were computed with the use of regression analysis.

In vitro cytotoxicity assay

A standard 4 hr ^{51}chromium (Cr) release assay was used to assess the cytotoxic reactivity of activated LN cells. Briefly, 2 x 10^6 tumor cells as target cells were labeled with 100 μCi sodium chromate-^{51}Cr (Dupone, N. Billerica, MA) at 37°C for 1.5 hr.

Labeled target cells (10^4) were incubated with various numbers of effector cells in 0.2 ml of CM in 96-well U-plates and incubated at 37°C for 5 hr. The supernatant was collected by the Titertek Collecting System (Flow Laboratories, McLean, VA) and counted in a gamma counter. The percentage cytolysis was calculated as:

$$\frac{\text{experimental cpm - spontaneous cpm}}{\text{maximal cpm - spontaneous cpm}} \times 100$$

Depletion of T cell subsets in vivo

Two hybridomas producing rat IgG2b monoclonal antibodies (mAb) directed at murine CD4 (GK 1.5, L3T4) and CD8 (2.43, Lyt 2.2) T cell antigens were obtained from the American Type Culture Collection (Rockville, MD). The mAb were produced as ascites fluid from sublethally irradiated (500cGy) and cyclophosphamide treated (100 mg/kg) DBA/2 mice. Mice were administered 0.2 ml of ascites i.v. in 1 ml HBSS. This procedure has been previously shown to be effective in producing long-term T cell subset depletion (9).

Results

Characterization of the immunogenicity of MT-901

This report documents the immunological host response to this newly established murine mammary tumor system, MT-901. By FACS analysis, MT-901 cells express MHC class I molecules but not class II (see Figure 1). The minimum reliable tumorigenic cell inoculum given s.c. was 10^4 cells. At a dose of 10^6 s.c., tumor cell irradiation of $\geq$ 5,000 cGy eliminated their tumorigenicity (data not shown). The immunogenicity of this tumor was assessed in several ways. The first approach was by the traditional immunization and challenge method. Animals were inoculated with irradiated 10^6 MT-901 tumor cells for immunization purposes. At least 2 weeks later, 10^6 viable tumor cells were administered at a separate s.c. site and tumor growth observed. As summarized in Table 1, irradiated tumor cells induced a protective systemic response in 45% of mice. The dose of irradiation did not appear to alter the antigenicity of the tumor. Based upon these findings, we have thus classified this tumor as weakly immunogenic.

Utilizing an adoptive immunotherapy model, we evaluated the ability of MT-901 to elicit tumor reactive pre-effector cells in draining LN. Mice were inoculated with MT-901 tumor cells in the flank and 9 days later had TDLN excised for anti-CD3/IL-2 activation. The antitumor reactivity of these cells was assessed by the adoptive transfer into mice bearing MT-901 or Renca pulmonary metastases. As shown in Table 2, MT-901 TDLN were effective in mediating the specific regression of MT-901 tumor, but not the Renca tumor. In vitro analysis of MT-901 TDLN cells revealed the tumor-specific release of IFNγ and GM-CSF only in response to MT-901 tumor cells, and not to Renca cells (Figure 2). In the same assay, control or naive lymphoid cells did not release cytokines in response to tumor simulation.

The ability of MT-901 to induce pre-effector cells in TDLN was compared to other lymphoid organs. MT-901 was inoculated s.c. into the flank of animals

followed 9 and 20 days later with the harvesting of TDLN, spleens and mesenteric LN for anti-CD3/IL-2 activation. The antitumor reactivity of these cells was assessed by the adoptive immunotherapy of 3-day lung metastases. As summarized in Table 3, nine days of tumor growth resulted in a systemic immune response with pre-effector cells retrievable from all tested lymphoid organs. Day 9 TDLN proved to be superior to spleen and mesenteric LN as a source of pre-effector cells. With longer tumor growth extending to 20 days, TDLN atrophied and were not adequate for anti-CD3/IL-2 culture. Both splenic and mesenteric LN cells revealed an absence of pre-effecter cells which documented a tumor-induced suppression phenomenon.

Tumorigenicity of genetically modified MT-901

We examined the tumorigenicity of MT-901 genetically modified to express the co-stimulatory molecule B7-1 or to secrete transgenic GM-CSF and IL-12 cytokines. A stably expressing B7-1 clone (MT-9B14, Figure 3) was inoculated s.c. in a group of normal mice and tumor growth compared with another group that received wild-type tumor. As shown in Figure 4, expression of transgenic B7-1 resulted in decreased tumorigenicity with 2 of 5 mice rejecting tumor completely. In a similar fashion, two GM-CSF secreting clones, MT-9G1 (240 ng/10^6 cells/24h) and MT-9G2 (40 mg/10^6 cells/24h) were examined and found to exhibit tumorigenicity (Figure 4). In the latter experiment, 0 of 6 mice inoculated with MT-9G1 and 2 of 5 mice inoculated with MT-9G2 rejected the tumor cells. In an immunization/challenge model similar to the description in Table 1, the immunogenicity of these clones after irradiation were compared to wild-type tumor. In a previous study, we had demonstrated that irradiated melanoma tumor cells transduced to secrete GM-CSF will continue to express transgenic cytokine for at least 7 days in vitro (14). As shown in Table 4, neither B7-1 expression nor GM-CSF secretion resulted in superior immunization against a subsequent tumor challenge compared to wild-type tumor alone.

In a separate experiment, we examined the tumorigenicity of a transfected clone MT-901.IL-12-4 which expressed IL-12 at a level of 4 ng/10^6 cells/24h of cytokine. As shown in Figure 5, s.c. inoculation of 10^6 cells resulted in initial tumor growth with subsequent rejection in all 5 mice. However, subsequent s.c. challenge of these mice with 10^6 wild-type tumor cells grew in all the animals.

Induction of pre-effector TDLN cells by genetically modified tumors

We next examined the immunogenicity of the cytokine transgene modified tumors for their ability to sensitize TDLN cells for adoptive immunotherapy. As summarized in Table 5, various tumor cells (MT-901, MT-9B14, MT-9G1 and MT-9G2) were inoculated s.c. and TDLN harvested 9 days later for anti-CD3/IL-2 activation. The antitumor reactivity of these cells were assessed by the adoptive transfer into mice bearing 3-day established MT-901 pulmonary metastases. In all 3 experiments, MT-9G1 which expressed the highest level of transgenic GM-CSF was significantly better than wild-type tumor in the induction of tumor reactive TDLN. The lower cytokine secreting MT-9G2 clone exhibited no difference as compared to the wild-type tumor in sensitizing TDLN cells. The B7-1 expressing MT-9B14 clone was significantly more efficacious than wild-type tumor in the induction of pre-effector cells in only 1 of 2 experiments; and, was not better than MT-9G1.

In a separate experiment, we evaluated the IL-12-transfected clone, MT-901.IL-12-4 which completely abrogated tumorigenicity in the immunocompetent host. Utilizing irradiated (5,000 cGy) tumor cells, we inoculated 10^7 cells s.c. of both wild-type or the IL-12-transfected clone. This enabled us to administer a larger antigen dose as well as achieving a higher level of IL-12 secretion to be elaborated locally (i.e., 40 ng/10^6 cells/24h). Table 6 shows that this clone did not elicit pre-effector TDLN cells differently from wild-type tumor. This was assessed at two different doses of transferred effector cells as a means of quantifying therapeutic efficacy on a per cell basis.

In vitro immune reactivity of GM-CSF secreting MT-901

We proceeded to investigate the in vitro immune reactivity of the GM-CSF secreting clones compared with wild-type tumor. Utilizing a standard 4 h ^{51}Cr release assay, we observed that MT-9G1 and MT-9G2 activated TDLN cells to be more lytic against MT-901 targets compared to wild-type TDLN which were non-lytic (Figure 6). The MT-9G1, which expressed more GM-CSF than MT-9G2, induced TDLN cells which manifested greater MT-901 lysis compared with MT-9G2 TDLN cells (Figure 6). Utilizing a cytokine release assay, we detected higher levels of IFNγ released by MT-9G1 and MT-9G2 TDLN cells as compared with MT-901 TDLN cells (Figure 7). The MT-9G1 TDLN cells also appeared more reactive in the release of IFNγ than MT-9G2 TDLN cells.

In vivo depletion of CD4+ and CD8+ T cells after adoptive transfer

We next examined the role of $CD4^+$ and $CD8^+$ T cells in mediating tumor regression after adoptive transfer. MT-9G1 TDLN cells were harvested and activated in vitro as before. Immediately after the adoptive transfer of 6×10^7 cells, mAb to deplete $CD4^+$ or $CD8^+$ T cells was administered. Rat immunoglobulin (RIg) was used as a control antibody. Select groups of mice received IL-2 while others did not. As summarized in Table 7, depletion of $CD4^+$ cells resulted in the abrogation of tumor regression whereas $CD8^+$ depletion did not. This was evident even in the presence of exogenously administered IL-2. In prior tumor models utilizing sarcomas, we have reported that $CD4^+$ cells were involved in tumor regression after adoptive transfer by secreting IL-2, and could be replaced by the exogenous administration of IL-2 (9). The MT-901 tumor model is different than those previous models since $CD8^+$ T cells were not required to mediate tumor regression. In addition, it appears that $CD4^+$ cells were responsible for the antitumor reactivity observed in this model which was not related to the elaboration of IL-2 by this cell subpopulation.

Therapeutic efficacy of MT-9G1 TDLN cells in the treatment of advanced tumor

Lastly, we examined the in vivo tumor reactivity of MT-9G1 TDLN cells in the treatment of advanced 10-day pulmonary metastases. Groups of animals were treated with IL-2 alone, or IL-2 plus MT-9G1 TDLN cells or MT-901 TDLN cells 10 days past i.v. tumor inoculation. As shown in Figure 8, MT-901 TDLN cells (8×10^7 cells/animal) did not significantly prolong survival compared with control groups that received no treatment or IL-2 alone. Mice that received MT-9G1 TDLN cells plus IL-2 had significantly prolonged survival compared with groups of mice that received no treatment of IL-2 alone ($p<0.05$ Wilcoxon test).

Discussion

In this study, we have extensively evaluated the host immune response to a syngeneic mammary adenocarcinoma tumor model, MT-901. This tumor was found to be weakly immunogenic by virtue of its ability to induce systemic immunity in 55% of mice inoculated with irradiated tumor cells. The subcutaneous growth of this tumor elicited sensitized T cells in different lymphoid organs which were capable of mediating the regression of established pulmonary metastases in an adoptive transfer model. Utilizing this model, we examined the immunogenicity of MT-901 tumor cells expressing transgenic GM-CSF, IL-12 and the co-stimulatory molecule B7-1.

The co-stimulatory molecules B7-1 and B7-2 have been reported to act as second signals to T cells which, in conjunction with antigen stimulation via the T cell receptor, results in the induction of host immunity (19). In animal studies, it has been shown that a series of weakly immunogenic tumor cells transfected to express B7-1 were more effective in the induction of systemic immunity compared with wild-type tumor cells (13). This latter observation was demonstrated in tumor vaccination and challenge experiments, and was not observed for several poorly immunogenic tumors. We generated an MT-901 clone which stably expressed transgenic B7-1. The clone manifested reduced tumorigenicity compared to the wild-type parental line. However, in vaccination and challenge experiments, the B7-1 transfectant was not more effective than wild-type tumor in the induction of systemic immunity. In addition, it was not significantly better than wild-type tumor in the induction of tumor reactive LN cells. By itself, the additional expression of transgenic B7-1 molecules by tumor cells did not appear to enhance T cell sensitization. We are exploring alternate approaches which may take advantage of B7-1 transgene expression, for example by combining it with the co-expression of different transgenes encoding cytokines (20) or other co-stimulatory molecules.

We proceeded to evaluate two cytokines with different immunopotentiating properties. The first was GM-CSF, a cytokine which has been shown to be a powerful adjuvant in tumor vaccination approaches. Dranoff et al. reported that the local elaboration of transgenic GM-CSF was significantly better than several other cytokines in promoting systemic immunity with B16 melanoma tumor vaccinations in a syngeneic animal model (11). In the present study, we show that GM-CSF is useful as an adjuvant in the induction of T cells with tumor reactivity in vivo. Compared to wild-type tumor, GM-CSF-transfected tumor cells resulted in enhanced cytotoxicity and cytokine release of TDLN cells in response to tumor antigen. These observations were evident despite our inability to demonstrate an increased immunogenicity of the GM-CSF secreting tumor cells by the vaccination and challenge experiments. The adjuvant effect of GM-CSF was clearly related to the level of transgenic cytokine elaborated, since a lower level secreting GM-CSF clone was shown to be less effective as the high level secreting clone in the generation of immune T cells. We hypothesize that the GM-CSF elaboration resulted in the accumulation of antigen presenting cells, notably dendritic cells, in the tumor and draining LN which facilitated T cell sensitization. The observations with the MT-901 extend our previous studies demonstrating that GM-CSF elaboration by a poorly immunogenic tumor can promote the sensitization of T cells effective in adoptive immunotherapy (14). In the latter study, the poorly immunogenic D5 melanoma tumor which lacked MHC class I and II molecules was incapable of stimulating T cells in the syngeneic C57BL/6 host unless inoculated with an immune adjuvant such as GM-CSF. From both studies, it is apparent that GM-CSF elaboration can promote T cell sensitization to both weakly and poorly immunogenic tumors in different strains of mice.

The second cytokine we examined was IL-12. IL-12 has proinflammatory properties and is known to modulate Thl helper T cells to secrete IL-2 and IFNγ.

Exogenous administration of IL-12 in animals has been demonstrated to confer potent antitumor effects against tumors of diverse immunogenicity (21). In a B16 tumor model, the paracrine secretion of IL-12 by genetically modified fibroblasts admixed with tumor cells was found to enhance active specific immunization (22). We established a stably transfected IL-12-expressing clone of MT-901 which manifested significantly reduced tumorigenicity. All mice injected with 10^6 transfected tumor cells rejected the inoculation; however, rechallenge with wild-type tumor demonstrated no evidence of systemic immunity. This may have been related to diminished tumor antigen exposure secondary to the reduced tumorigenicity. In an effort to evaluate T cell induction by the IL-12 transfected tumor cells, we injected a 10-fold higher tumor inoculum s.c. and compared it with a comparable number of wild-type tumor to assess draining LN function. Both cell inoculums were irradiated which did not alter the antigenicity of the tumor cells, and allowed a larger dose of tumor antigen to be administered. In addition, the amount of IL-12 elaborated by 10^7 cells reached 40 ng/10^6 cells/24h which was similar to amounts found effective as an immune adjuvant in other reports (23). We found, however, that at this level of IL-12 secretion, we could still not demonstrate an upregulated T cell sensitization beyond that evident with wild-type tumor. Several possibilities may potentially account for this observation. The first may be a quantitative phenomenon of inadequate amounts of IL-12 secretion. We did however attempt to compensate for this as noted above. The second may be the mechanisms operative in tumor eradication with the MT-901 model. We have found that MT-901 tumor regression is apparently mediated primarily via $CD4^+$ T cells. The antitumor reactivity promoted by IL-12 therapy has been reported in many cases to be mediated by $CD8^+$ cells (24). We are currently evaluating the mechanisms by which $CD4^+$ cells in this model mediate tumor regression in vivo.

In summary, we have established an alternate animal model to assess immunotherapeutic approaches against a weakly immunogenic mammary carcinoma. The MT-901 expresses tumor-specific antigens as assessed in adoptive transfer studies of sensitized T cells. We have defined the relative immunogenicity of tumor transfectants that express three different immunoregulatory molecules: GM-CSF, IL-12 and B7-1. We demonstrated that GM-CSF was an effective adjuvant in the sensitization of T cells against the MT-901 tumor.

Figures

1. Flow cytometric analysis of MHC class I and II molecule expression on MT-901 cells.
2. Tumor-specific cytokine release by MT-901 TDLN cells stimulated by irradiated MT-901 or Renca tumor cells in vitro. Cells were cultured in the presence of 60 IU/ml IL-2 with supernatants harvested 48 h later for measurement of IFNγ and GM-CSF.
3. Flow cytometric analysis of B7-1 co-stimulatory molecules on MT-901 and MT-9B14.
4. Tumor growth of MT-9G1, MT-9G2 and MT-9B14 was measured after inoculation of 10^6 cells subcutaneously.
5. Tumor growth of MT-901.IL-12-4 was measured after inoculation of 10^6 cells subcutaneously.
6. Cytotoxicity of MT-901, MT-9G1 and MT-9G2 TDLN cells to wild-type tumor targets. Tumor lysis was measured at different effector to target (E/T) ratios.
7. IFNγ cytokine release of MT-901, MT-9G1 and MT-9G2 TDLN cells in response to wild-type tumor.
8. Survival of mice with advanced 10-day established pulmonary metastases after adoptive immunotherapy. Some groups of mice were injected with 8×10^7 MT-901 or MT-9G1 TDLN cells plus 30,000 IU of IL-2 twice a day for 8 doses.

Table 1: Growth of MT-901 in Animals Immunized with Irradiated Wild-Type Tumor

Immunization with Irradiated Tumor (cGy)[1]		No. of Animals with Tumor after Challenge[2]			
		Exp 1	Exp 2	Exp 3	Total (%)
A.	None	5/5	5/5	5/5	15/15 (100)
B.	5,000	3/5		1/5	
C.	10,000		3/5		
D.	15,000		4/5		
E.	Combined B, C, D				11/20 (55)

1 Animals inoculated with 10^6 irradiated tumor s.c.

2 Animals challenged with 10^6 non-irradiated tumor s.c. at least 14 days after immunization. Data represent no. of animals with tumor/total no. immunized animals.

Table 2: Specificity of MT-901 TDLN Cells in the Therapy of Pulmonary Metastases

	Treatment		Pulmonary	Mean No. of Pulmonary
	MT-901 TDLN[1]	IL-2[2]	Metastases	Metastases (SEM)
A.	-	-	MT-901	247 (5)
B.	-	+	MT-901	205 (5)
C.	+	+	MT-901	2 (2)[d]
D.	-	-	Renca	249 (3)
E.	-	+	Renca	175 (92)
F.	+	+	Renca	225 (35)

1 Mice were inoculated with 1.5×10^6 MT-901 cells s.c. and TDLN cells harvested 9 days later for activation by the anti-CD3/IL-2 method. Six $\times 10^7$ activated TDLN cells were then administered to animals with established 3-day pulmonary metastases.

2 30,000 IL-2 i.p. twice daily after cell transfer for 8 doses.

3 Lungs were harvested 14 days after tumor inoculation.

4 $p<0.01$ compared to groups A and B.

Table 3: Pre-effector Cell Response in Different Lymphoid Organs Induced by the Growth of MT-901

	Treatment		Mean No. of Lung Metastases (SEM)[3]	
	Cells[1]	IL-2[2]	6×10^7	1.5×10^7
A.	None	-	235 (9)	
B.	None	+	218 (17)	
C.	Day 9 TDLN	+	7 (1)[4,5]	209 (20)
D.	Day 9 spleen	+	39 (6)[4]	234 (13)
E.	Day 9 mesenteric LN	+	105 (45)[6]	236 (15)
F.	Day 20 spleen	+	204 (210)	182 (42)
G.	Day 20 mesenteric LN	+	>250	212 (24)

1 Mice were inoculated with 1.5×10^6 MT-901 s.c. and lymphoid organs harvested 9 or 20 days later for activation by the anti-CD3/IL-2 method.

2 Same as Table 2.

3 Activated TDLN cells were administered i.v. to animals with 3-day MT-901 pulmonary metastases at two different doses (6×10^7 or 1.5×10^7 per animal).

4 $p<0.001$ compared to groups A and B.

5 $p<0.01$ compared to group D.

6 $p<0.05$ compared to groups A and B.

Table 4: Growth of MT-901 in Animals Immunized with Irradiated MT-9B14, MT-9G1 or MT-9G2.

	Tumor Used for Immunization[1]	No. of Animals with Tumor after Challenge[2]	
		10^6 cells	5×10^6 cells
A.	None	5/5	5/5
B.	MT-901	2/5	1/5
C.	MT-9B14	1/5	5/5
D.	MT-9G1	2/5	4/5
E.	MT-9G2	5/5	5/5

1 Animals inoculated s.c. with 10^6 tumor cells irradiated with 5,000 cGy.

2 Animals challenged with 10^6 or 5×10^6 MT-901 non-irradiated tumor cells 21 days after immunization. Data represent no. of animals with tumor/total no. of immunized animals.

Table 5: In Vivo Efficacy of TDLN Cells Derived from Animals Inoculated with GM-CSF Transduced Tumors

	Source of TDLN[1]	In Vivo IL-2[2]	Mean No. of Pulmonary Metastases (SEM) Exp 1	Exp 2	Exp 3
A.	None	-	>250	>250	>250
B.	None	+	206 (43)	210 (39)	235 (15)
C.	MT-901	+	123 (42)[3]	85 (33)[3]	46 (13)[3]
D.	MT-9G1	+	16 (4)[4]	37 (18)[4]	5 (1)[5]
E.	MT-9G2	+	107 (50)[3]	127 (36)[3]	-
F.	MT-9B14	+	56 (26)[3]	197 (33)	-

1 Mice were inoculated with 1.5×10^6 tumor cells s.c. and TDLN cells harvested 9 days later for activation by the anti-CD3/IL-2 method. Two $\times 10^7$ (Exp 1 and 2) or 4×10^7 (Exp 3) activated TDLN cells were administered i.v. to animals with established 3-day MT-901 pulmonary metastases.

2 Same as Table 2.

3 $p<0.05$ compared to groups A and B.

4 $p<0.05$ compared to groups A, B, C, E, F.

5 $p<0.05$ compared to groups A, B, C.

Table 6: In Vivo Efficacy of TDLN Cells Derived from Animals Inoculated with IL-12 Transfected Tumors

Treatment[1]		Mean No. of Pulmonary Metastases (SEM)[2]	
TDLN	IL-2	4×10^7	10^7
A. None	-	212 (24)	
B. None	+	170 (32)	
C. MT-901	+	13 (4)[3]	52 (15)[3]
D. MT-901.IL-12-4	+	10 (5)[3]	34 (14)[3]

1 Mice inoculated with 10^7 irradiated (5,000 cGy) tumor cells and TDLN harvested in similar fashion as in Table 2. IL-2 administered as in Table 2.

2 Activated TDLN cells were administered i.v. to animals with 3-day MT-901 pulmonary metastases at two different doses (4×10^7 or 10^7 per animal).

3 $p<0.05$ compared to groups A and B.

Table 7: In vivo depletion of $CD4^+$ and $CD8^+$ T cells after adoptive transfer of MT-9G1 TDLN cells

	Treatment[1]		Antibody	Mean No. of Pulmonary
	MT-9G1 TDLN	IL-2	Depletion[2]	Metastases (SEM)
A.	-	-	-	>250
B.	+	-	RIg	94(27)[3]
C.	+	-	anti-CD4	243(10)
D.	+	-	anti-CD8	86(27)[3]
E.	-	+	-	>250
F.	+	+	anti-CD4	230 (27)
G.	+	+	anti-CD8	48(40)[3]

1 Mice were administered 6×10^7 TDLN cells generated as in Table 5. IL-2 administered as in Table 2.

2 0.2 ml of antibody was administered i.v. shortly after adoptive transfer of cells.

3 $p<0.001$ compared to groups A, C, E, F.

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FIGURE 1

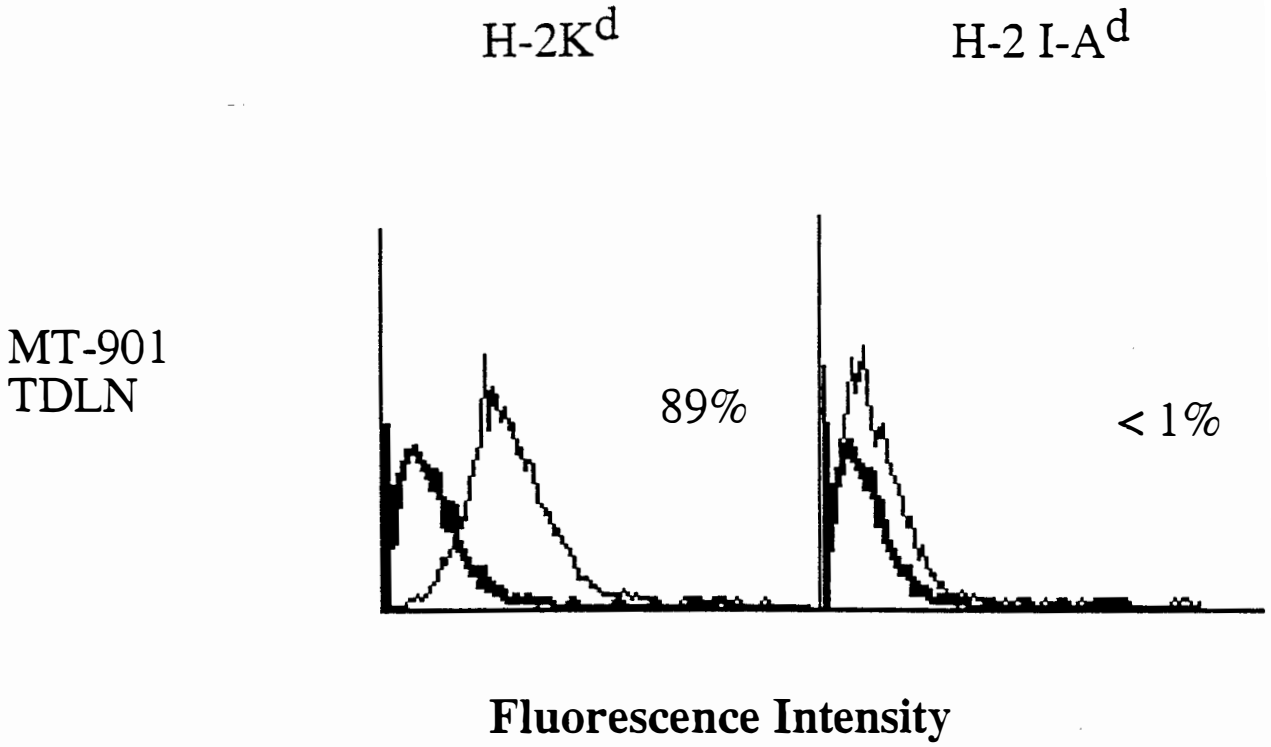


FIGURE 2

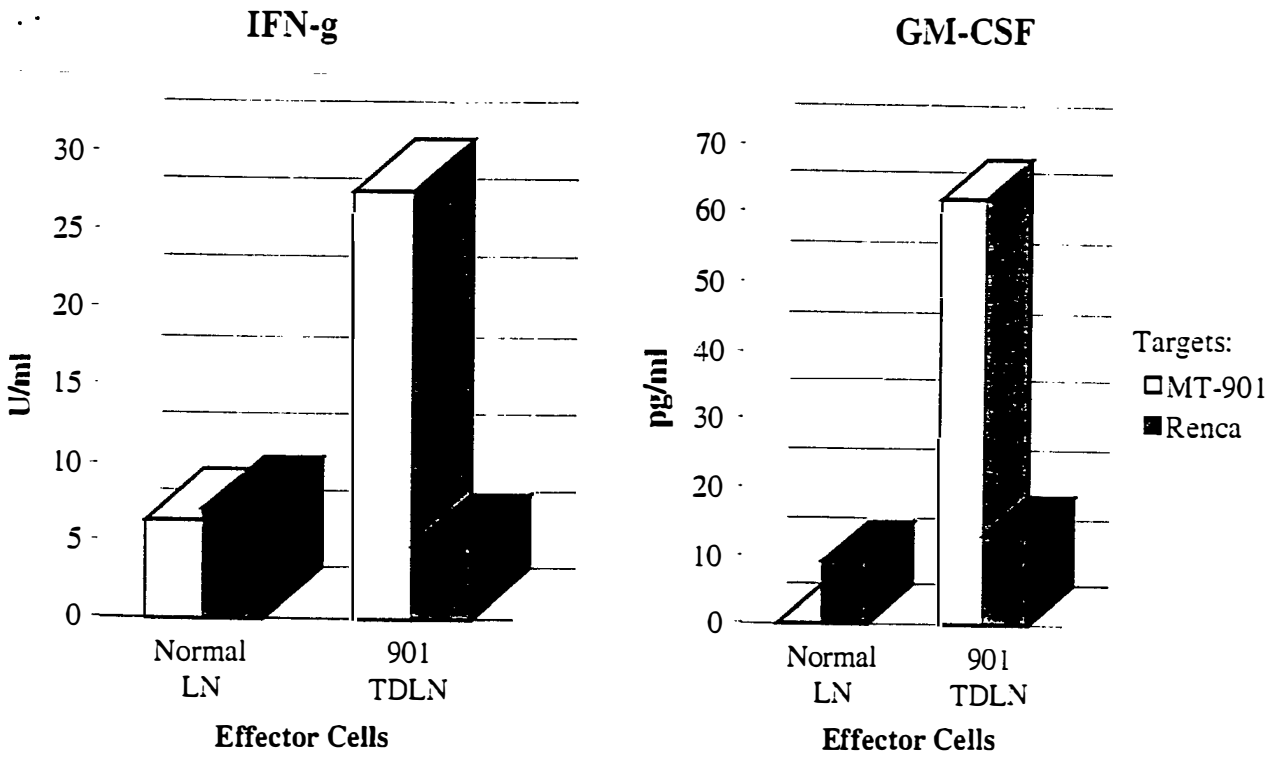

IFN-g
30
25
20
15
10
5
0
U/ml
Normal LN
901 TDLN
Effector Cells
GM-CSF
70
60
50
40
30
20
10
0
pg/ml
Normal LN
901 TDLN
Effector Cells
Targets:
MT-901
Renca

FIGURE 3

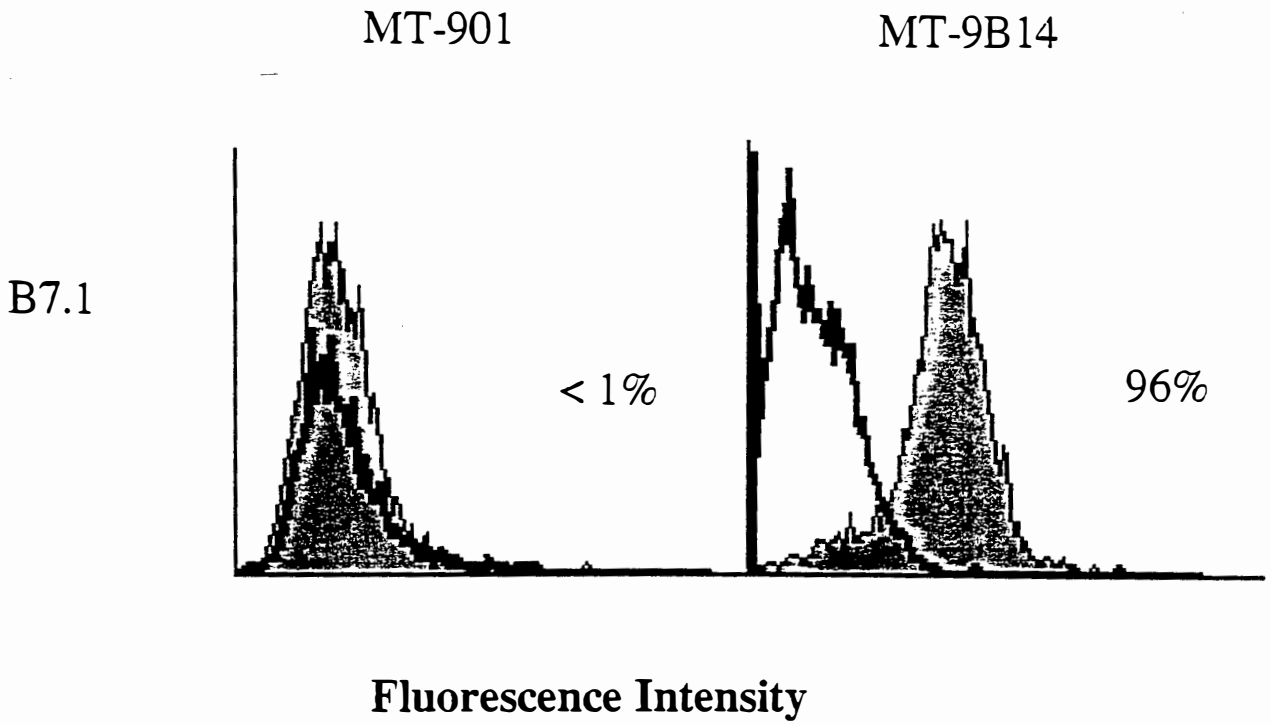


FIGURE 4

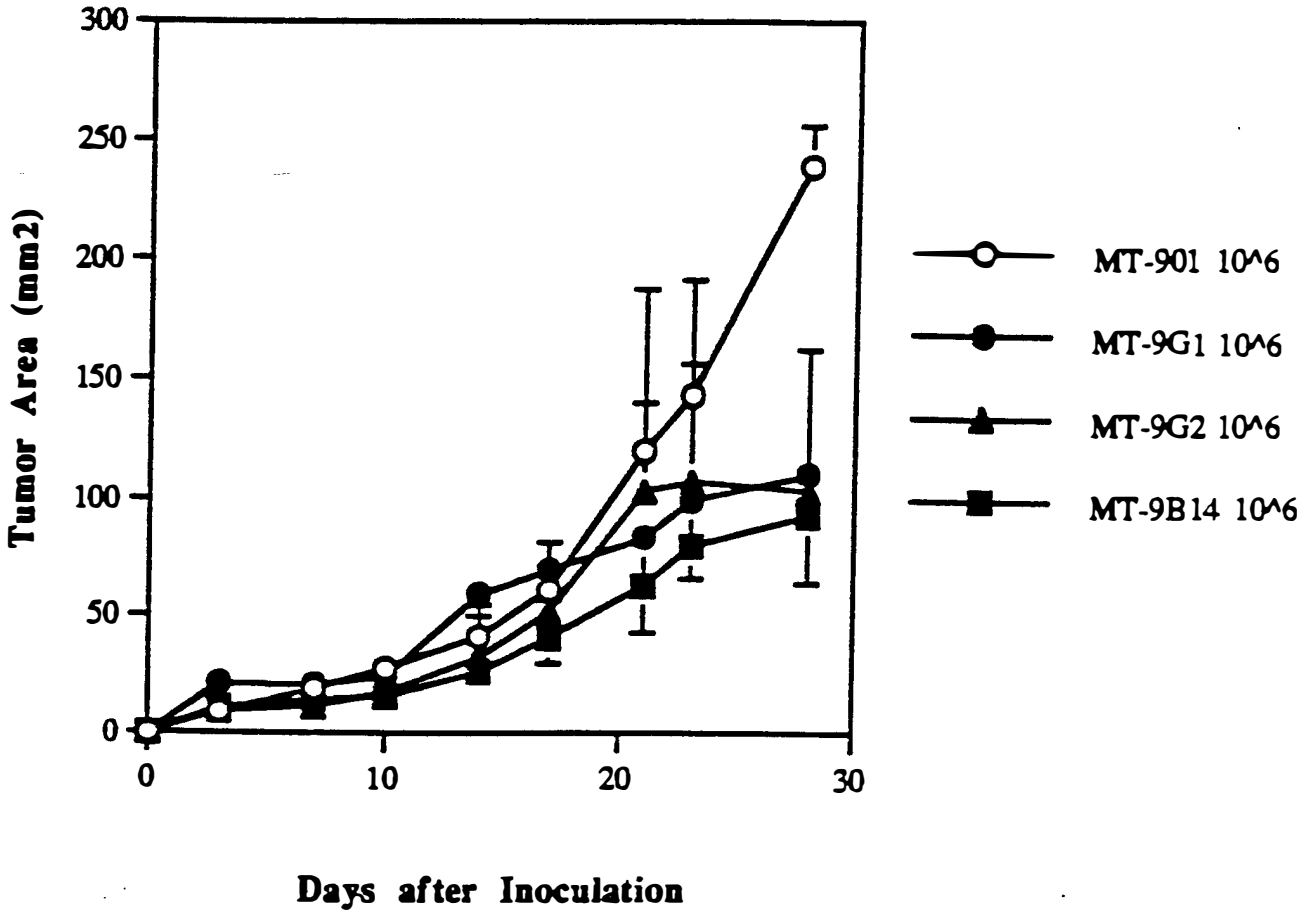

300
250
200
150
100
50
0
Tumor Area (mm2)
0
10
20
30
Days after Inoculation
MT-901 10^6
MT-9G1 10^6
MT-9G2 10^6
MT-9B14 10^6

FIGURE 5

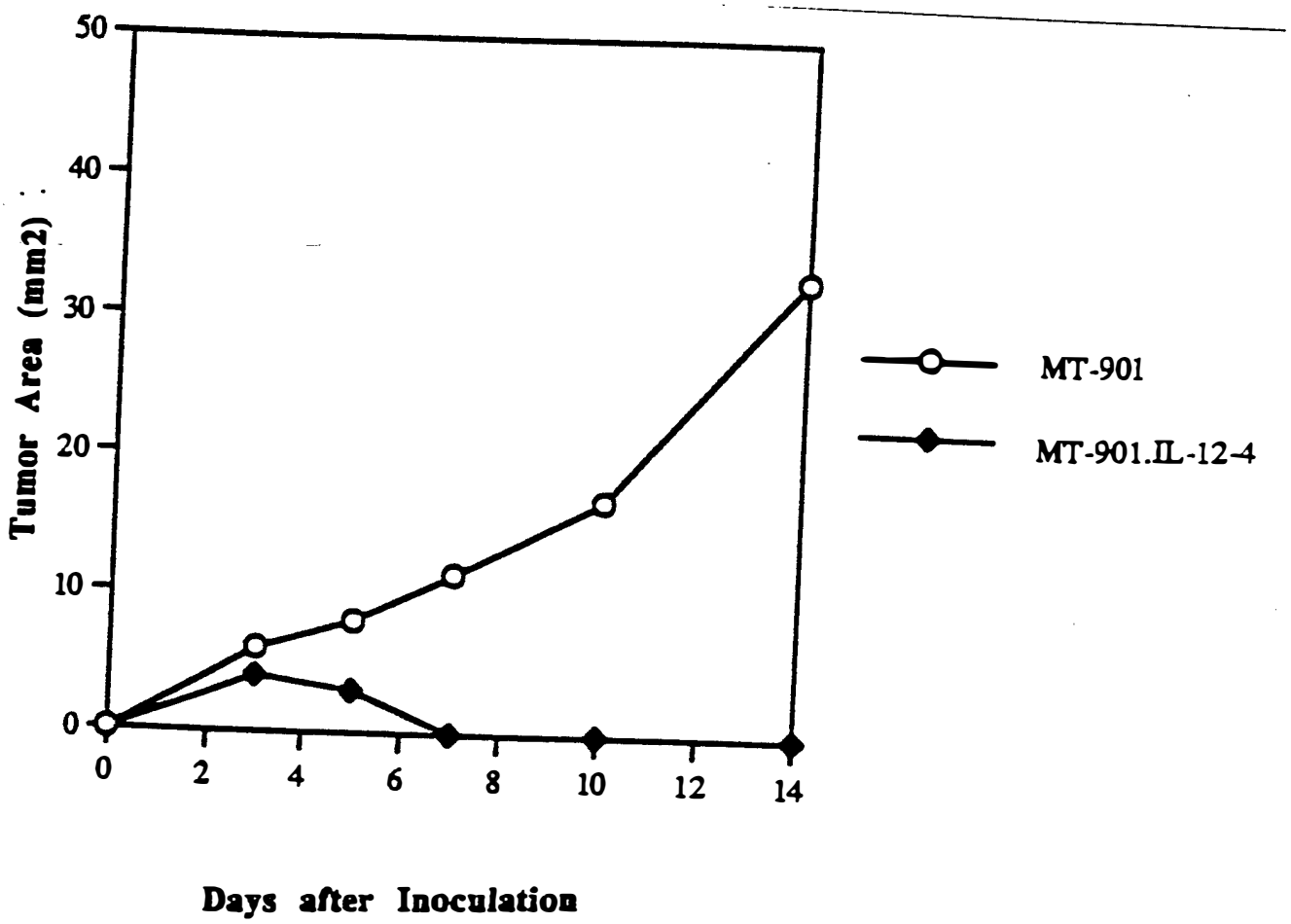

50
40
30
20
10
0
Tumor Area (mm2)
0
2
4
6
8
10
12
14
Days after Inoculation
MT-901
MT-901.IL-12-4

FIGURE 6

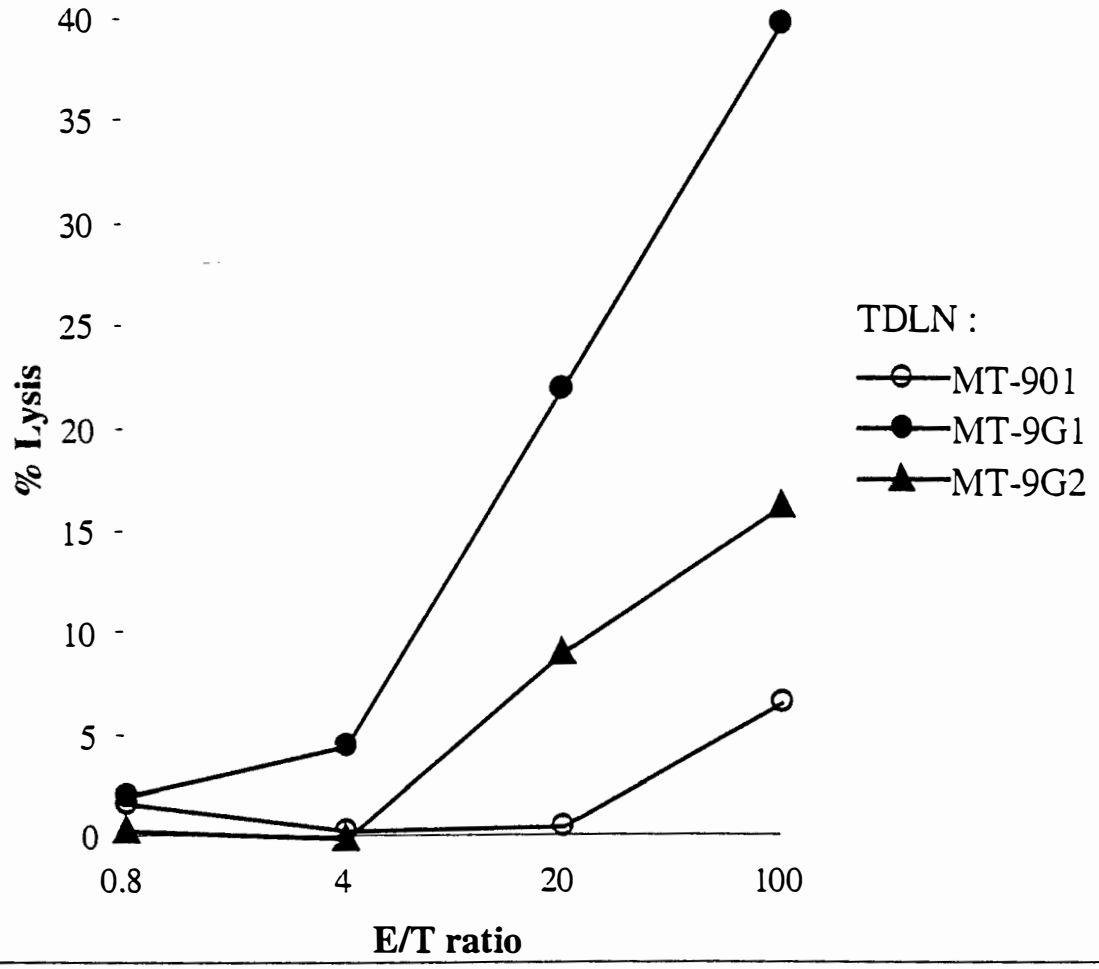

40
35
30
25
20
15
10
5
0
% Lysis
0.8
4
20
100
E/T ratio
TDLN :
MT-901
MT-9G1
MT-9G2

FIGURE 7

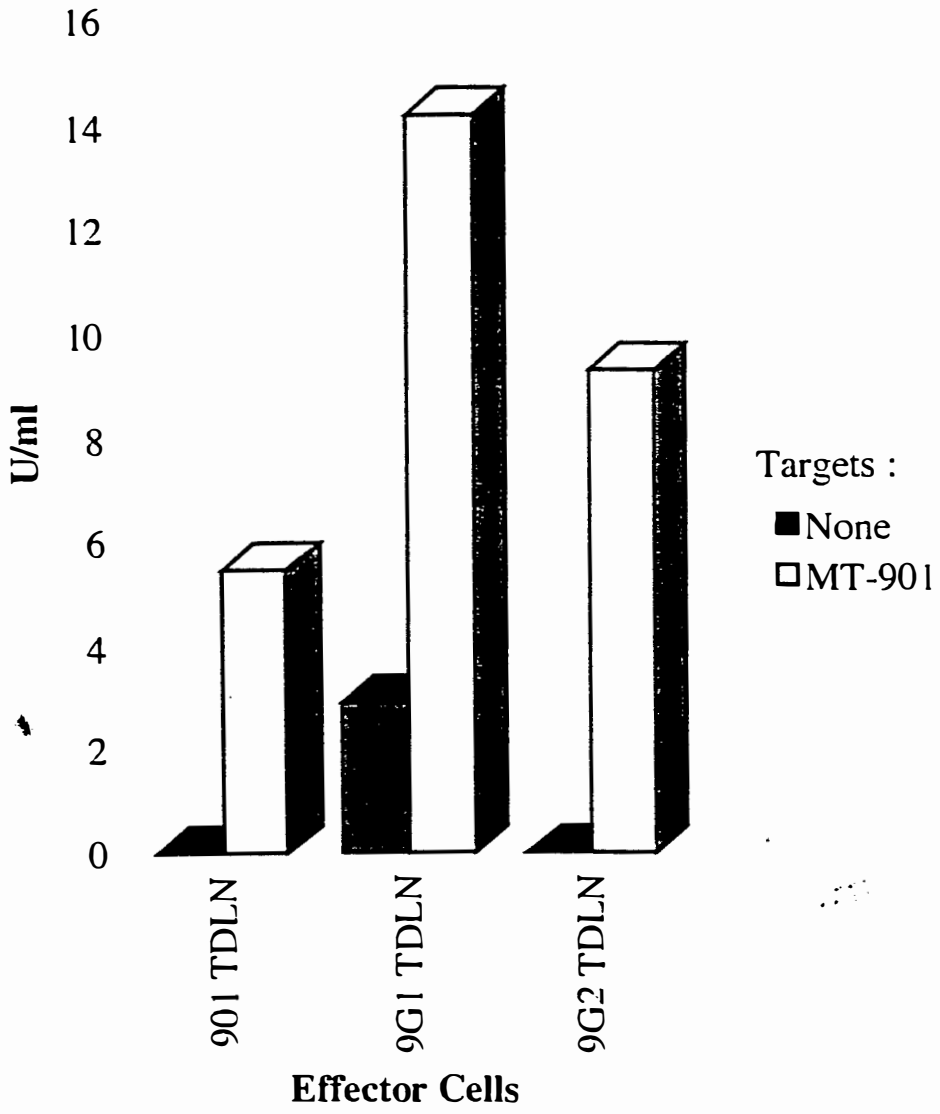
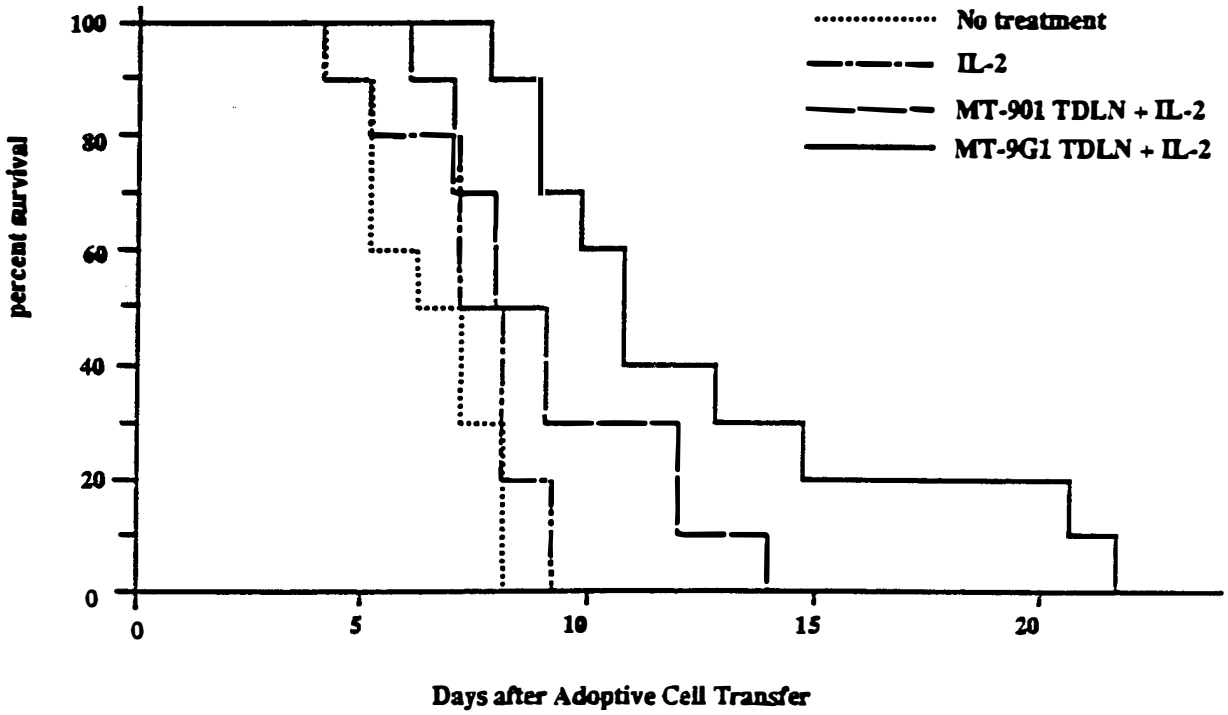

16
14
12
10
8
6
4
2
0
U/ml
901 TDLN
9G1 TDLN
9G2 TDLN
Effector Cells
Targets :
None
MT-901

FIGURE 8


No treatment
IL-2
MT-901 TDLN + IL-2
MT-9G1 TDLN + IL-2
percent survival
100
80
60
40
20
0
0
5
10
15
20
Days after Adoptive Cell Transfer