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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
α(1,3)galactosyl epitopes (αGal) expressed on membrane glycoproteins and glycolipids are the major target of the human hyperacute rejection response observed when organs are transplanted from nonprimate donor species. Expression of the α(1,3)galactosyltransferase gene [α(1,3)GT] by breast cancer cells will lead to their direct *in vivo* destruction by anti-αGal antibodies and complement, and boost the immune response to other tumor antigens. *In vitro* transduction of MCF-7, and T47D human breast cancer cells with an HSV amplicon vector resulted in expression of the suicide gene as detected by specific binding of labeled IB-4 isolectin. Transduced breast cancer cells were susceptible to antibody and complement-mediated cytolysis as demonstrated in serum killing assays with 50% human serum. The α(1,3)GT knockout mouse is analogous to humans in terms of expression of αGal epitopes. A syngeneic murine breast cancer cell line from α(1,3)GT knockout mice has been produced by chemical induction (BR340)that is being used to demonstrate protective immunity when cells are *ex vivo* transduced and implanted into αGal stimulated knockout mice.

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INTRODUCTION

From 1999 to 2001, an estimated 125,500 women have died from breast cancer despite all currently available therapies (1-3). New approaches are urgently needed to combat this deadly disease. Gene therapy is among the current experimental strategies for patients with cancer who have failed standard therapy, yet evidence for success in the more than 100 gene therapy trials to date is limited (4). New candidate genes and delivery methods must be pursued. This proposal exploits the α(1,3) galactosyltransferase [α(1,3)GT] gene for cancer therapy by converting transduced breast cancer cells into vaccines to stimulate systemic anti-cancer immunity. Strong immunologic reaction to xenotransplants from lower mammals into humans is based upon immunity to the α(1,3) GT gene product. Rejection of organ and tissue xenotransplants (5) occurs because antibodies (Ab) in human serum to the α (1,3) Galactosyl epitope (α Gal) exist (6) and represent nearly 1% of total human serum Ab (7). This pre-existing human Ab is the basis for complement-mediated hyperacute rejection of xenotransplants (8). The strategy of this proposal is to use the murine α(1,3)GT gene as a therapeutic transgene to induce hyperacute rejection of human breast cancer like that associated with xenotransplants. Since expression of α(1,3)GT modifies multiple glycoproteins and glycolipids on the cell surface, the human immune system should have an increased opportunity to detect and process breast cancer specific antigens. Our novel HSV amplicon vectors offer highly efficient gene transfer and expression of the α(1,3)GT gene. Herpes vectors are extremely attractive since they efficiently transduce nondividing cells (G_0 stage) and the majority of human breast tumor cells are not actively dividing. These vectors transduce a wide variety of human adenocarcinomas at much lower multiplicity of infection (MOI) than required for retroviral or adenoviral vectors that are currently used in the majority of gene therapy approaches.

BODY

Task 1. Demonstrate efficacy of HE7αgal1 amplicon vector in human breast cancer cells.

1a. Transduce human breast cancer cells with HE7αgal1 vector and demonstrate αgal epitope expression on the cell surface.

Status: Completed

MCF-7 human breast adenocarcinoma cells, T47D human breast ductal carcinoma cells, and SKBR3 human breast adenocarcinoma cells were selected for testing the *in vitro* efficacy of the HE7αgal1 amplicon vector. All cells were maintained at 37°C in a 5% CO_2 incubator. The growth medium for MCF-7 cells consisted of Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen-Life Technologies, Carlsbad, CA) supplemented with bovine insulin (0.1 mg/ml) (Sigma, St. Louis, MO), and 10% FBS (Invitrogen-Life Technologies). T47D cells were maintained in RPMI 1640 medium (Invitrogen-Life Technologies) supplemented with bovine insulin (0.1 mg/ml) (Sigma), 0.1 mM nonessential amino acids (Sigma), 0.45% Glucose, and 10% FBS (Invitrogen-Life Technologies). SKBR3 cells were maintained in McCoys 5A medium (Invitrogen-Life Technologies) supplemented with 10% FBS (Invitrogen-Life Technologies), and 10% conditioned medium.

CgalΔ3 helper herpes virus is deleted for the IE3 gene and was propagated on the permissive cell line E5 (African Green Monkey Kidney), which contains a stably integrated IE3 gene. For propagation, 90% confluent cultures of E5 cells were infected with 0.1 moi of CgalΔ3 helper virus. Following incubation at 37°C for 72 hr, cells were harvested and subjected to three rounds of freeze/thawing. Virus was concentrated by centrifugation at 28,000 rpm for 1 hr and resuspended in 10% sucrose. Packaged HE7αgal1 amplicon was produced by transfecting the amplicon into E5 cells prior to CgalΔ3 propagation. Both helper virus and HE7αgal1 amplicon were titered on VA13 human fibroblast cells.

Breast cancer cells were cultured overnight in a 24 well plate (Corning Co., Corning, NY). Cells were washed and infected with HE7αgal1 vector at 3 moi in 200 µl of Opti-MEM (Invitrogen-Life Technologies). Plates were rocked to mix for 2 hr, and 300 µl of complete medium was added. Lectin staining for αGal epitopes, or serum killing assays were performed 24 hr post-transduction.

HE7αgal1 transduced tumor cells were stained with *Griffonia simplicifolia* (Vector Laboratories, Burlingame, CA) IB_4 isolectin conjugated to fluorescein isothiocyanate (FITC). α(1,3)Gal epitopes are specifically bound by the GS IB_4 isolectin. Transduced cells were washed with 1x HBSS (Invitrogen-Life Technologies), then incubated under dark conditions at room temperature with 200 □l of a 10□l/ml

solution of GS IB_4 isolectin diluted in 1x OptiMEM (Invitrogen-Life Technologies) for 10 minutes. Cells were washed with 1x HBSS, and viewed under 488 □m wavelength light. Labeled cells were observed for lectin binding using a Nikon Diaphot 300 Fluorescent microscope (Nikon Inc. Melville, NY), and photographed using a Nikon CoolPix995 digital camera (Nikon Inc.).

MCF-7, T47D, and SKBR3 human breast cancer cells were all successfully transduced by HE7αgal1 amplicon vector, and expression of αGal epitopes on surface proteins was detected by FITC- IB_4 isolectin staining (Fig 1).

1b. Demonstrate human breast cancer cell sensitivity to human serum after HE7αgal1 vector transduction and that the cytotoxicity is secondary to complement activation.

Status: Completed

Propagated HE7αgal1 vector was treated with psoralen and UVA light (PUVA) to reduce helper virus cytotoxicity but retain high transgene expression prior to use in assays. Psoralens are polycyclic planar molecules that form covalent, cyclobutane-type linkages. Previous studies applying crosslinking methods with psoralen and UVA completely inactivated virus. The appropriate PUVA dose induces DNA crosslinks in the vector and inhibits replication in E5 cells while retaining reporter gene expression. Psoralen was diluted to 1 μg/ml in HBSS and mixed with HE7αgal1 vector at room temperature for 30 minutes. Vector was then exposed to UVA ($2Kj/m^2$) for 5.5 minutes. Vector was then titered on VA13 cells as before.

Serum killing assays were performed in 96-well round bottom tissue culture plates. Transduced cancer cell lines were trypsinized, concentrated by centrifugation, and suspended in Opti-MEM. Cells were counted using Trypan Blue Exclusion, and 50 μl containing 1×10^6 cells was seeded into wells. Frozen human serum was thawed on ice and added 1:1 with cell suspensions to create a 50% dilution of human serum with transduced cancer cells, and cells were incubated for 1 hr at 37°C in a 5% CO_2 incubator. Cells were transferred to a flow cytometry culture tube, washed with 4.0 ml of Opti-MEM, and incubated with GS IB_4 isolectin (FITC) and Propidium iodide for 15 minutes under dark conditions. Cells were washed with 4.0 ml of HBSS, and suspended in 1.0 ml of HBSS for analysis by flow cytometry.

Flow cytometry was performed using a Coulter Epics Atra Flow Cytometer (Coulter Corp, Miami, FL) for two color analysis, gated for approximately 10,000 events. Human serum killing of transduced human breast cancer cell lines is shown in Fig 3.

1c. Complete and publish manuscript concerning *in vitro* transduction and complement mediated destruction of human breast cancer cells.

Status: In progress

Task 2. Demonstrate efficacy *in vivo* of HE7αgal1 vector in murine breast cancer model in α(1,3)GT knockout mice.

2a. Generate sygeneic breast tumor cell lines derived from α(1,3)GT knockout mice.

Status: Completed

The αGal epitope is expressed in all mammals except humans and Old World primates. Lowe et al., produced an α (1,3)GT knockout strain of C57/BL6 mice by interrupting the α (1,3)GT gene (9). This mouse strain lacks a functional α (1,3) GT gene. It has been shown that these mice, like humans, produce anti-αgal antibody, albeit much less than in humans (10). When primed with rabbit red blood cells, which express approximately 10^6 αgal epitopes per cell, anti-□gal antibody titer rises to that of levels found in humans (11). The only murine tumor line known to be devoid of αgal expression was the C57/BL6 derived melanoma B16F0 and its derivatives (12). We induced the formation of tumors in the αgal knockout mice through multiple injections of 3-methylcholanthrene (3-MC), 9,10-dimethyl-1,2-benzanthracene (DMBA), and 6α-methyl-17β-hydroxy-progesterone acetate (MD) all dissolved in olive oil. Forty-four tumors were collected and approximately 50% were successfully cultured. Primary histopathology demonstrated tumors ranging from squamous cell carcinoma of the dermis, to adenocarcinoma of the small intestine, and adenocarcinoma of the breast.

To confirm the absence of αgal expression, tumor cells were stained with the αgal epitope specific FITC-labeled IB_4 lectin. All cell lines tested were negative upon staining. Expression of αgal epitopes on these tumor cells is critically important if they are to be used as tumor vaccines. Furthermore, gene transfer by an HSV-1 viral vector had to be established in these cells in order to use this gene delivery vehicle in both *in vitro* and *in vivo* experiments. Therefore, The BR340 α(1,3)GT Knockout breast cancer cell line was transduced with HE7αgal1, and αgal epitope expression was measured by IB_4 lectin binding. This murine cell line expressed high levels of αgal epitopes (Fig 2).

2b. Generate protective immunity against breast tumor cell challenge by preimmunization with *ex vivo* HE7αgal1 vector transduced breast *cancer* cells

Status: In progress

In order to analyze protective immunity associated with agal in knock-out mice, we had to establish growth characteristics of the newly developed breast tumor line BR340. Determination of cell dose,

volume and duration was vital to defining an in vivo model. However, we have spent much time in characterizing BR340with limited success.

After establishing BR340 in culture by multiple passage (greater than 30) we began to analyze the in vivo growth characteristics. Tumor cells were introduced subcutaneously at a concentration of $1x10^6$ per 100 µl, and $5x10^6$ per 100 µl in Hanks Balanced Salt Solution. It was noted that tumors developed after three days and continued to maximal size by day 10. Where upon the tumors began regressing. By day 25 all animals were devoid of tumors and remained that way for 90 days. During that time it was evident that the tumor cells appeared to be pleomorphic in culture, a common occurrence with DMBA induced tumors (13). In order to clone a subpopulation, we introduced $1x10^7$ cells per animal and conducted serial in vivo passages of resulting tumors. Each tumor was harvest before reaching 10 days, cultured and re-introduced into another animal. After five passages the tumor cells were expanded in culture and introduced into 8 mice. Unfortunately the response was similar, regression after 10 days in vivo.

We began a parallel investigation into the genetic background of the mice from which this tumor was derived, the same colony into which we were implanting BR340 cells. It became apparent that the transgenic knock-out mice were not backcrossed to yield an inbred strain. Moreover, the curators of this strain, from which we derived our colony after taking delivery of 2 female and 1 male confused their paperwork upon sending us the animals. Originally were agreed on only siblings, rather we received female mice of distant relation to one another and the male. Therefore, we found that the genetic background of C57/Bl6 x DBA/2 x 129SV produced to haplotypes of d or b. In lieu of that finding we haplotyped our tumor lines and nearly 400 mice from our colony. Anti-H2-K^d, Anti-H2-K^b, Anti-H2-D^b, Anti-H2-D^d antibodies (Pharmingen, San Diego, CA) were used on blood samples collect from the saphenous vein of mice. Also, these antibodies were used to analyze tumor cells by flow cytometry (Fig 4). We found that the BR340 tumor line belongs to the d haplotype. Furthermore, since the tumor was derived from a female mouse, it became apparent that tumor growth may be optimized in haplotype and sex match mice.

After several months of amplifying the colony, d haplotype female mice were used to assess the growth characteristics of BR340. Cells were introduced by subcutaneous inject as described above and followed for growth. Figure 5 demonstrates that the growth characteristics followed the same pattern as previously described.

In order to understand the basis behind the tumor rejection, a retroviral vector carrying the cytomegalovirus US11 gene was introduced to BR340 cells. US11 inhibits expression of the major histocompatibility complex I, a major mediator of graft rejection. Median fluorescence of BR340LUSN

decreased by 74% from parental and 90% from the control (null retroviral vector, LXSN) (Fig 6). However, when the cells were introduced into haplotype and sex matched animals, the cells failed to grow past 10 days.

At this point we began to investigate several simultaneous approaches in order to develop this tumor model. First we subjected the cells to stringent serum starvation (SS) conditions. BR340 cells were trypsinized and reseeded in a T-175 flask at a concentration of $2x10^6$ cells and allowed to attach overnight in complete media supplemented with 10% fetal bovine serum (FBS). The follow day the growth media was changed to complete media with 0.1% FBS and allowed to grow for 13 days. The media was changed to complete media with 2.0% FBS and allowed to grow for another 3 days whereupon the cells were further recovered in complete media with 10% FBS. In a parallel approach, BR340 was subject to 1mCu I^{131} for 24hrs which resulted in the killing of approximately 90% of the cells. The cells were recovered and subjected to 1.5mCu I^{131} for 48hrs. Approximately 98% of the cells were killed and the remaining cells were recovered in complete media.

Both of these modified cell lines were introduced into mice and followed for 90 days. All tumors but one regressed by day 14. Animal D708 received a subcutaneous injection of SS modified BR340 at $1x10^6$ cells. By day 30 the tumor was approximately $550mm^3$. We harvested the tumor and cut it in half for in vivo propagation and in vitro amplification. Five tumor fragments were introduced subdermally in matched mice and the remaining tumor was digested and culture in complete media. After a week amplification, the BR340.D708 cells were introduced by subcutaneous injection into the mammary fat pad of 8 matched mice and tumor growth was followed.

The implanted tumor fragments failed to grow larger tumors whereas the subcutaneous injections produced tumors that again regressed by day 14. A common observation with BR340 in vivo, as was true from the primary tumor, was that as the tumor grew the center began to necrotize. By day 10 noticeable cell death was observed as the the tumor began to take on a gelatinous appearance. On the belief that the tumor may be dying because of a lack of growth factors, we investigated the use of a gel used to help establish growth of tumors in vitro and in vivo. We mixed MatriGel (BD BioSciences, Bedford, MA) with BR340.D708 cells and injected as before. This time we noted sustained tumor growth to day 25 before regression began.

Further analyses by flow cytometry demonstrated that BR340 expresses the α chain of the estrogen receptor. We reasoned that since the tumor grew very well in tissue culture without a requirement for hormonal supplement, that growth in vivo may not be affected by supplemented estrogen. To prove that,

we implanted estrogen tablets (1.6mg/tablet 60 day slow release) subdermally and 24hrs later injected BR340 cells as above. No gain in growth rate or duration was observed with this treatment.

We are currently attempting to clone individual subclones of BR340 by inducing spheroid formation on soft agar. Miller had demonstrated that drug induced tumors are often not monoclonal and that subclones can demonstrate immunodomminance over other subclones (13). Therefore, we feel it may be of some value to attempt to subclone this tumor line and investigate the subclones for in vivo growth. Also, we have further mutagenized BR340 using short wavelength UVC radiation. One million cells each were subject to various doses of UVC and plated in complete media. At 200J/m2, for example, only 4 colonies survived the treatment. Each treatment will be repeated on the respective cells and each population will be introduced into 8 animals each. Resulting tumor will be propagated both in vitro and in vivo. Furthermore, since DMBA binds DNA directly, it is possible to further mutagenize the cells with this drug. Therefore, 0.1mg of DMBA will be mixed with media and applied to $1x10^6$ cells and allowed to grow for 1 wk. The process will be repeated and resulting cells will be introduced to animals as described above.

Finally, we are beginning to develop new breast tumor in 4-6 wk old mice by direct injection into the mammary fat pad with DMBA dissolved in olive oil. Resulting tumors will be analyzed by histopathology, cultured in vitro, and serially passaged in vivo immediately following tumor harvest.

2c. Destroy subcutaneous breast cancer tumors by direct injection on HE7αgal1 vector *in vivo*
Status: In progress

2d. Evaluate humoral and cellular immune responses to αgal epitope presentation by breast tumors
Status: In progress

2e. Complete and publish manuscript concerning *in vivo* immunization and protection using HE7αgal transduced murine breast cancer cells derived from α(1,3)GT knockout mice.

Status: In progress

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that HE7αgal1 vector can effectively transduce human breast cancer cell lines (MCF-7; T47D; and SKBR3), and the generated murine breast cancer cell line (BR340).
- Demonstrated expression of the αGT transgene in breast cancer cell lines transduced with HE7αgal1 vector by specific binding of FITC-labeled IB_4 lectin and fluorescent microscopy.
- Demonstrated sensitivity of human and BR340 murine breast cancer cell lines to human serum after HE7αgal1 transduction using 50% human serum in an *in vitro* assay.
- Generated a syngeneic murine breast cancer cell line derived from α(1,3)GT knockout mice (BR340) using chemical induction with 3-MC, DMB, and MD.
- Generated other syngeneic murine cancer cell lines (small intestine, squamous cell carcinoma, and fibrosarcoma) that will be very useful in future work using αGT as a therapeutic gene for cancer therapy.
- Determined potential new avenues of research based upon possible complement-inhibition and interference of serum killing of αGT expressing cancer cells.

REPORTABLE OUTCOMES

The research supported by this funding has generated the following reportable outcomes:

- A manuscript submitted to the journal Cancer Research "Immunity to the α(1,3)Galactosyl Epitope Provides Protection in Mice Challenged with Colon Cancer Cells Expressing α(1,3)Galactosyltransferase: A Novel Suicide Gene for Cancer Gene Therapy"

- A manuscript draft concerning generation of sygeneic tumor cell lines that can be used for development of cancer gene therapy protocols with α(1,3)GT

- Abstracts and presentations at:

    Komen Foundation "Reaching for the Cure – Innovations in Quality Care" 2001
    Oral Presentation – Breast Cancer Vaccine by Inducing Hyperacute Rejection

Department of the Army – Era of Hope Meeting 2002
    Poster Presentation – A Novel Therapy for Breast Cancer by Inducing Hyperacute Rejection

    2000 ASGT Annual Meeting – Eliciting Hyperacute Rejection of HSV α(1,3) Galactosyltransferase Transduced Tumors

    2000 ASGT Annual Meeting – Evidence for a Protective Immune Stimulation from α(1,3)Galactosyl Epitopes in Mice

    2001 AACR Annual Meeting – Generation of a Recombinant Adenovirus Carrying the Gene for α(1,3)Galactosyl Transferase, For use as a Suicide Gene in Cancer Immunotherapy

    2002 AACR Annual Meeting – Suicide Gene Expression by an HSV Amplicon Vector Using a Complementation System of an IE3-deficient Herpesvirus with a Recombinant Adenovirus Expressing the HSV-ICP4 Gene in Co-infected Cells.

- Support of Dissertation Degree work for co-invetigator Daniel Hellrung. Expected graduation date, 6/2003

- Submission of two proposals for clinical trial based upon α(1,3)GT cancer gene therapy.

CONCLUSIONS

The work supported by this funding has demonstrated the possibility of using the α(1,3)GT transgene as a potent effector for cancer gene therapy. The HE7αgal HSV vector that we have produced is able to successfully transduce human breast cancer cell lines that we have tested, and can also transduce the generated syngeneic murine breast cancer cell line. This is an important factor necessary for destruction of transduced cells by human serum. Our results conclusively show that human serum will kill α(1,3)GT expressing tumor cells *in vitro*. We have also shown *in vivo* that an immune response to the αGal epitope in α(1,3)GT knockout mice can provide protection when mice are challenged with an αGal-positive tumor cell line (submitted manuscript). All findings to date have supported the hypothesis that the α(1,3)GT gene is an excellent candidate for gene therapy for breast cancer.

A major objective of this work, was the generation of a syngeneic breast tumor cell line that could be used in this work to further test our hypothesis. This goal was achieved, in addition to the generation of tumor cell lines from other murine tissues. These cell lines will be invaluable for future work in the characterization and refinement of α(1,3)GT cancer gene therapy.

Difficulties have been encountered while attempting to use the generated BR340 murine breast cancer cell line for generating *in vivo* tumors. These difficulties may be due to the low tumorigenicity of the cell line, or perhaps these cells are highly immunogenic. As stated, we are attempting to overcome this problem by further mutagenizing the cell line. In order to generate data that can be used in support of the fundamental hyposthesis, until difficulties in growing the BR340 cell line are solved, we are considering using one of the other generated syngeneic tumor cell lines. Figure 7 shows the growth curve generated when the CA320M small intestine carcinoma cell line is injected subcutaneously into the α(1,3)GT knockout mouse. This tumor cell line is able to grow and generate subcutaneous tumors more readily, and could be temporarily used as a model.

We have generated data and new research materials (cell lines) through support of this work supports the original hypothesis of using the α(1,3)GT gene for gene therapy for breast cancer. And data demonstrate this hypothesis has a high potential for leading to an effective breast cancer gene therapy. Continued support and work will undoubtedly generate additional significant data leading to the stated goal.

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Figure 1.

Human Breast Carcinoma Transduced With HE7αgal1 and labeled with FITC IB_4 Isolectin

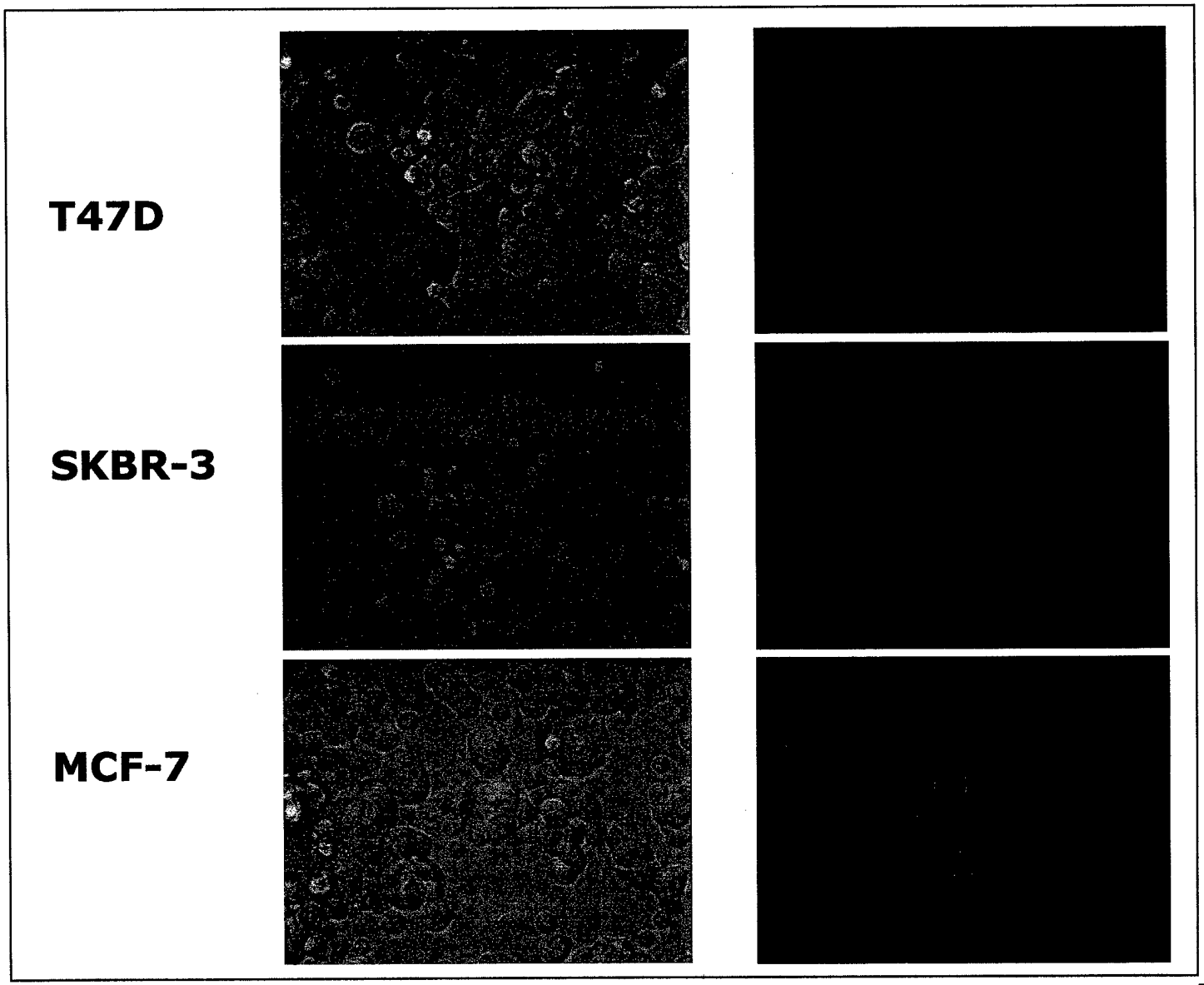


Figure 2.

Murine Breast Carcinoma Transduced With HE7αgal1 and labeled with FITC IB_4 Isolectin

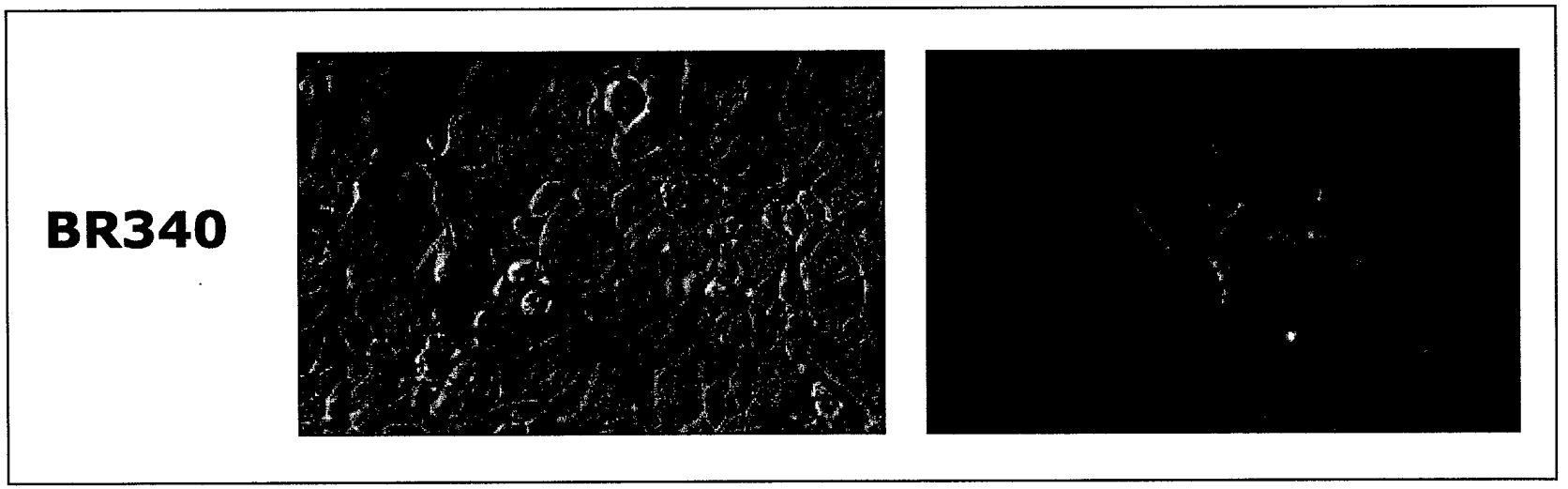


Figure 3

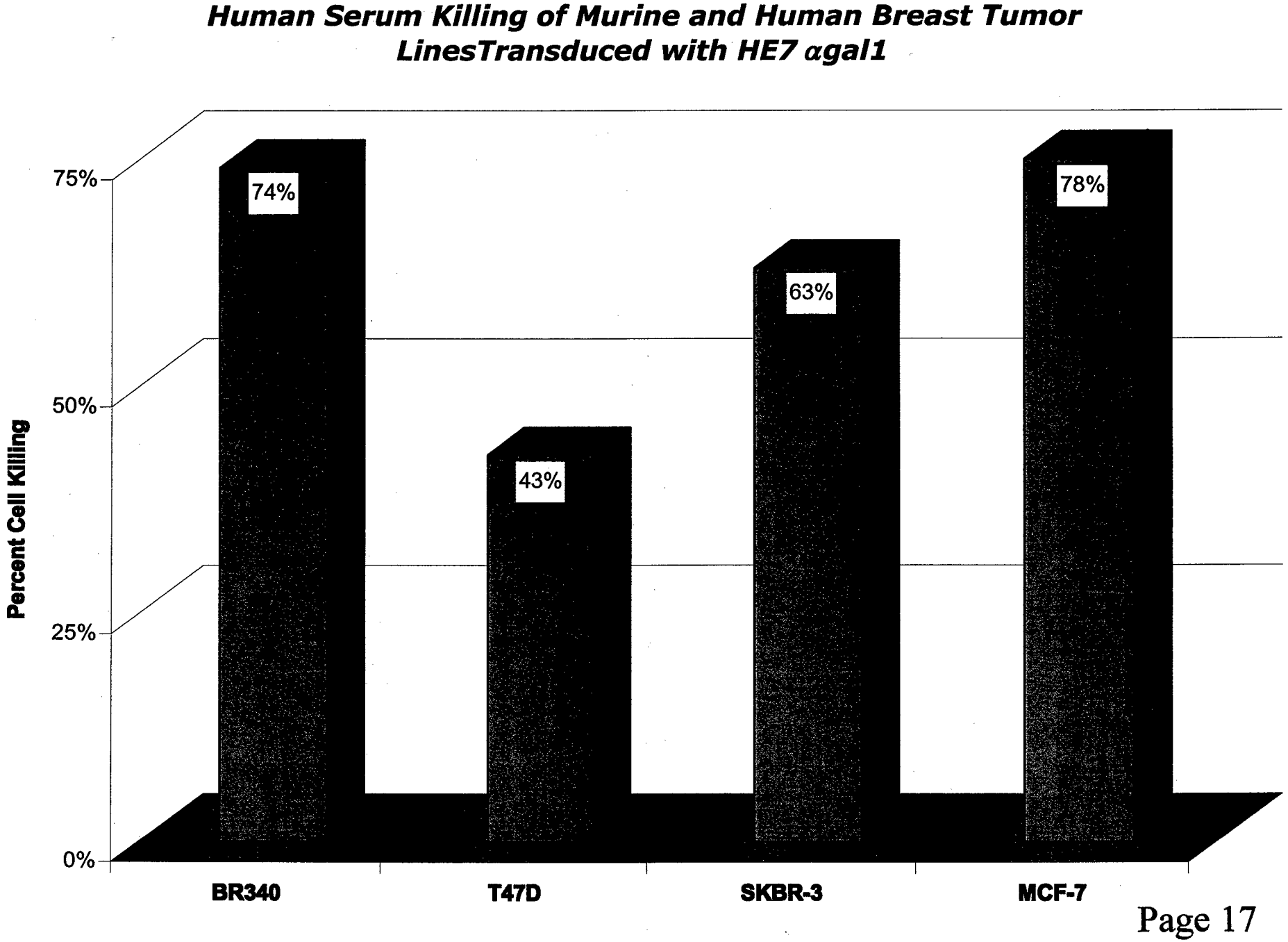

Human Serum Killing of Murine and Human Breast Tumor LinesTransduced with HE7 αgal1
Percent Cell Killing
75%
50%
25%
0%
74%
43%
63%
78%
BR340
T47D
SKBR-3
MCF-7

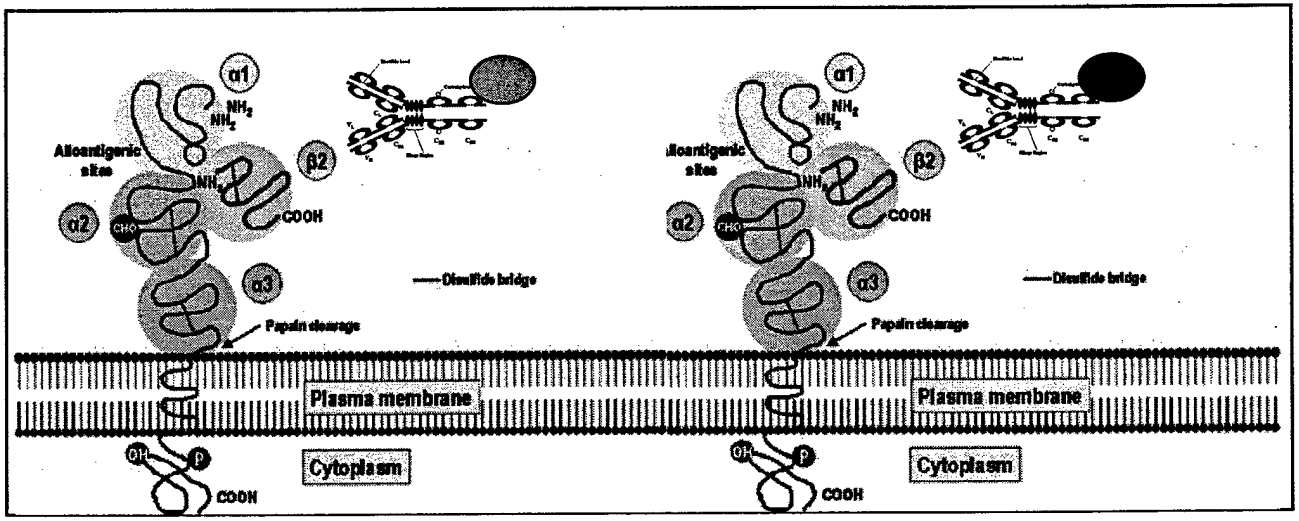
Figure 4.

Haplotyping

1. Isolate Blood

2. Label with FITC or PE conjugated anti-H2 Ab

3. HBSS Wash and View



Tumor	H2-K^d	H2-D^b	H2-K^k	H2-D^d
172	2	2	0	0
320M	2	2	0	0
260B	0	0	4	4
340	0	0	3	3

Figure 5

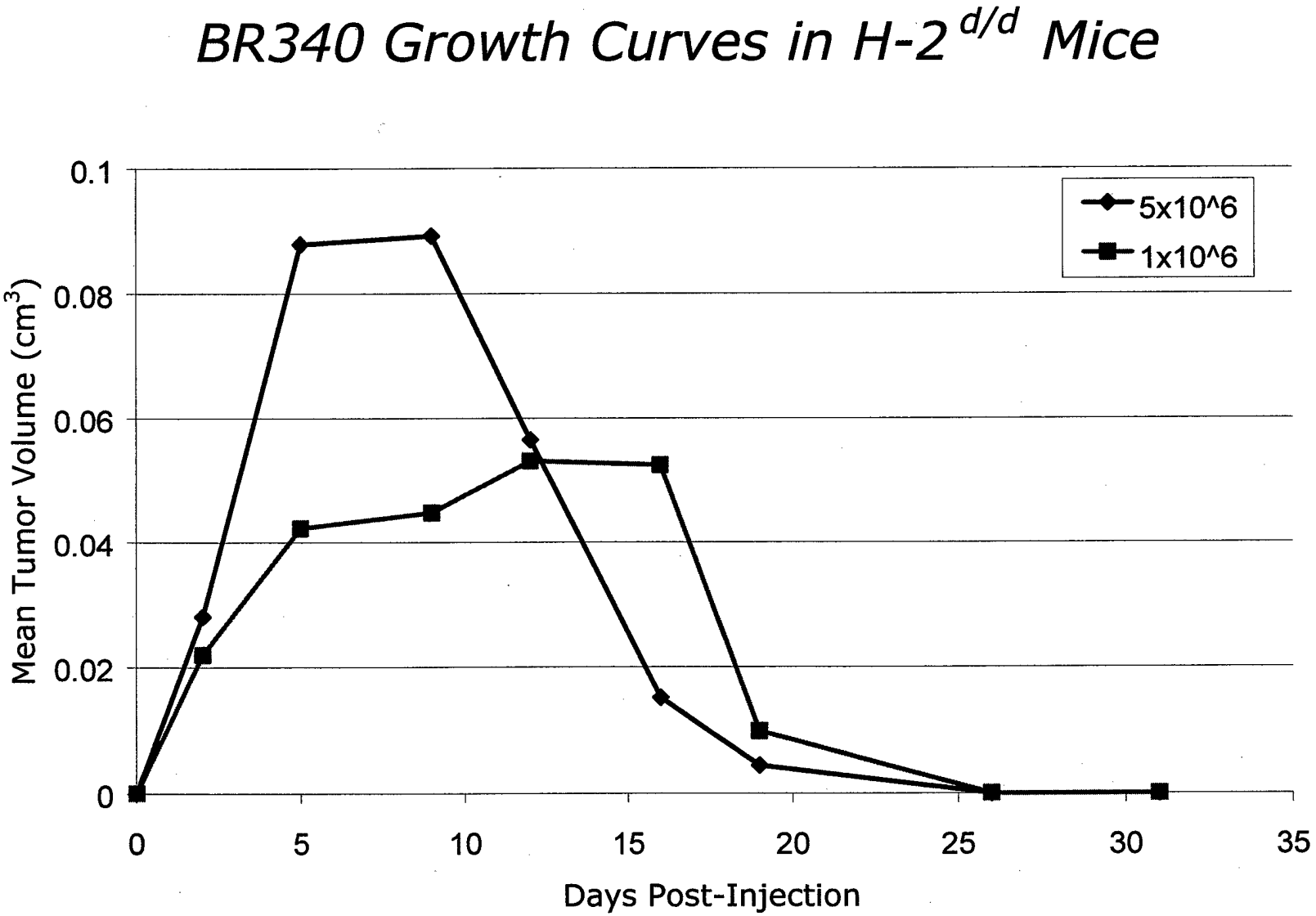

BR340 Growth Curves in H-2 d/d Mice
0.1
0.08
0.06
0.04
0.02
0
Mean Tumor Volume (cm3)
0
5
10
15
20
25
30
35
Days Post-Injection
5x10^6
1x10^6

Figure 6.

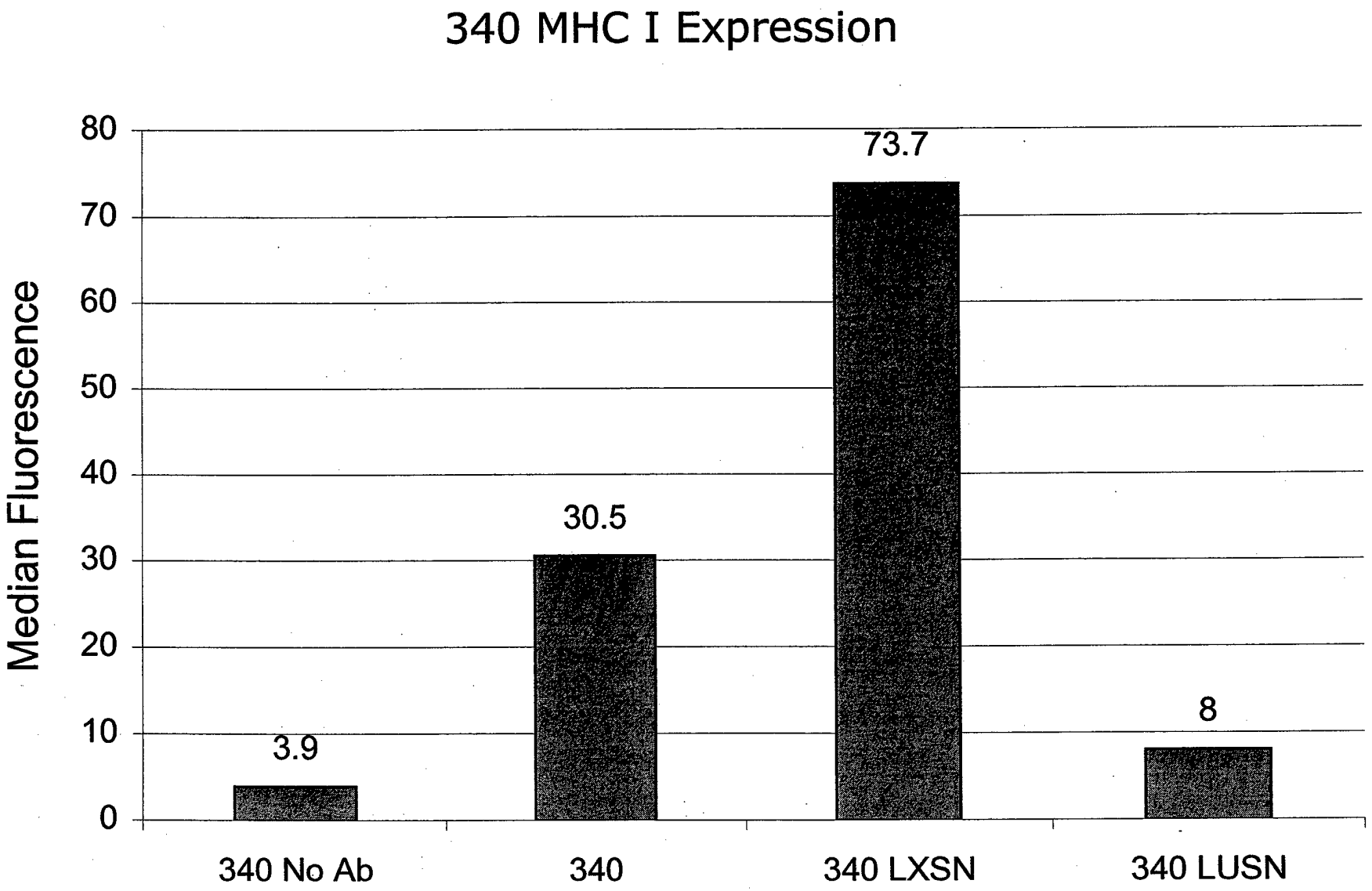
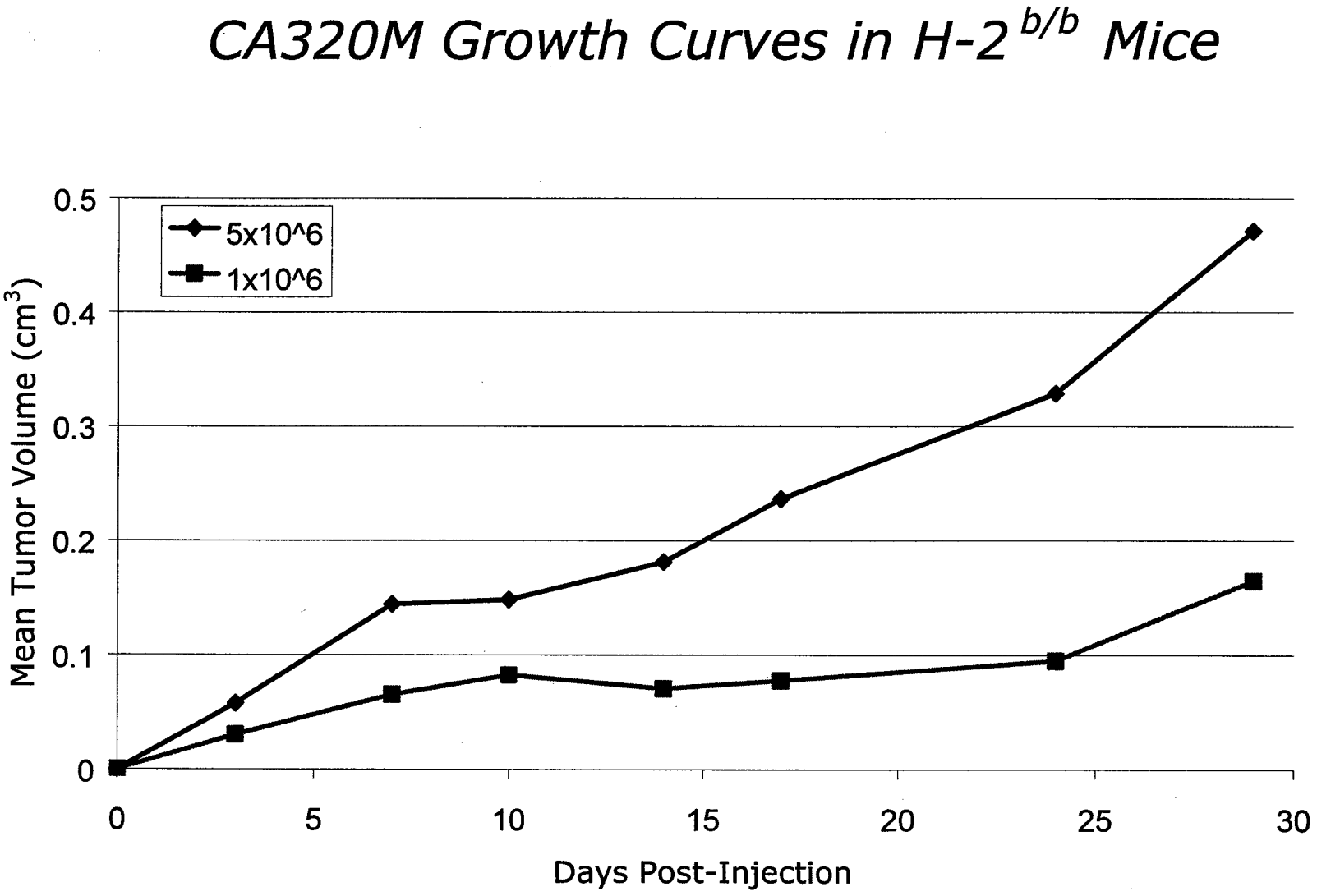

340 MHC I Expression
Median Fluorescence
80
70
60
50
40
30
20
10
0
3.9
30.5
73.7
8
340 No Ab
340
340 LXSN
340 LUSN

Figure 7


CA320M Growth Curves in H-2 b/b Mice
5x10^6
1x10^6
Mean Tumor Volume (cm3)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Days Post-Injection

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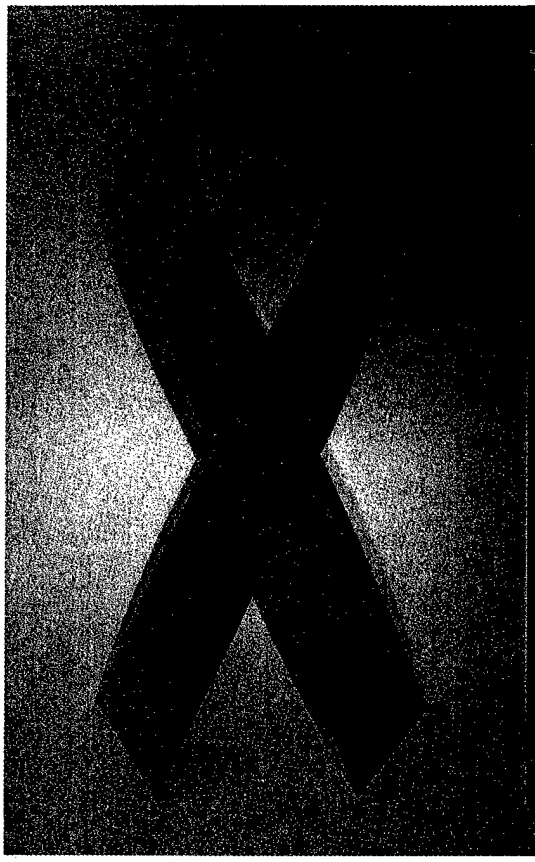

ABSTRACT BOOK

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Breast Cancer Vaccine by Inducing Hyperacute Rejection

Charles J. Link, Jr., MD
Human Gene Therapy Research Institute

In women with breast cancer, the immune system fails to recognize cancer within the body despite the fact that cancer cells produce many abnormal proteins. This research seeks to change breast cancer cells so that they are perceived as foreign tissue by the human body. A gene from a mouse is transferred by an artificial virus into breast cancer cells so the cells appear as if they come from a mouse. This causes the immune system to immediately attack the cancer cells (a process called hyperacute rejection).

Experimental procedures. The mouse gene has been cloned into a common form of virus. This virus infects human breast cancer cells easily. The infected breast cancer cells are then used as a vaccine. This system is being modeled in mice to generate data in order to initiate a human clinical trial.

Progress to date. The mouse gene has been placed into a variety of artificial viruses. A mouse model of breast cancer has been developed to test the vaccine.

Potential Outcomes. These experiments are being conducted to move the research into a clinical trial of this vaccine approach in women with breast cancer.

ORAL PRESENTATION - SUSAN G. KOMEN CONFERENCE

A NOVEL THERAPY FOR BREAST CANCER BY INDUCING HYPERACUTE REJECTION

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This project presents an innovative approach to breast cancer gene therapy that exploits a naturally occurring physiologic process in humans. We propose that the alpha(1,3)galactosyltransferase [a(1,3)GT] gene in fact represents an ideal unconventional "suicide" gene to induce destruction of the tumor, because the mediators of cell death are inherent in the human immune system and not dependent on pharmacological intervention. Strong immunological barriers to xenotransplants from lower mammals into humans can destroy a transplanted solid organ within minutes, a process termed hyperacute rejection. The expression of the murine a(1,3)GT gene results in the cell surface expression of a(1,3)galactosyl epitopes (agal) on membrane glycoproteins. These epitopes are the major target of the human hyperacute rejection response that occurs when organs are transplanted from nonprimate donor species. a(1,3)GT is expressed in all mammals including Mus musculus, but not in old world primates, apes or humans. We employed a novel Herpes amplicon vector (HE7agal1) that efficiently infects human solid tumor cells at low multiplicity of infection and permits high-level transgene expression. The anti-tumor effectiveness of a(1,3)GT gene transfer has now been show in vitro in human breast tumor cell lines with rapid killing after normal human serum exposure by complement lysis. The a(1,3)GT knockout mouse serves as the only small animal model analogous to humans in which to study the immune response to the agal epitopes. a(1,3)GT KO mice immunized with rabbit RBC (agal+) produced high-titer anti-agal Ab responses in all mice. When agal immunized mice were challenged by injection with agal positive tumor cells, 70% to 100% of the mice survived up to 30 days. Control normal mice (agal+) challenged with the same tumor died uniformly before day 19 after challenge. Therefore, the presence of anti-agal Ab was highly protective. Next, a(1,3)GT KO mice were administered carcinogens to generate a murine breast cancer cell line (agal negative) for an in vivo tumor model. A murine breast cancer cell line (agal negative) was derived from these mice. In conclusion, our data show effective use of the murine a(1,3)GT gene as a therapeutic transgene to induce hyperacute rejection of breast cancer.

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Eliciting Hyperacute Rejection of HSV α(1,3) Galactosyltransferase Transduced Tumors

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Abstract

Rejection of xenotransplants has been characterized by a major antigen, αgal, a product of the α(1,3) galactosyltransferase (αGT) gene. A golgi dependent enzyme, αGT, catalyzes the addition of a galactose from uridine diphosphate galactose (UDP-Gal) to the penultimate N-acetyl-lactosamine acceptor in an α (1,3) epitope (αgal) on glycoproteins and glycolipids. Moreover, mutations in this gene in humans, apes, and old world primates have resulted in an evolutionary divergence from all other mammals. Interestingly, in the former group, data suggests that 1% of circulating antibodies are directed against this carbohydrate epitope. It has been demonstrated that mimicking αgal epitopes are present on normal intestinal flora providing a possible explanation for such high anti-αgal titers. With this information we have sought to exploit this phenomenon in a gene therapy approach for the treatment of cancer. Therefore, human serum was collected from donors and frozen in aliquots to preserve complement factors. Fifty percent serum was mixed with MC38 αgal^+ murine colon carcinoma cells for 1 hour resulting in 98% killing. Based on this observation we used a herpes viral vector (HE7αgal1) system to deliver the murine αGT gene to various human tumor cell lines as well as a murine B16F10αgal^- melanoma cell line. After serum treatment as above, we noted a reduction up to 90% in cell viability against controls. In order to decipher the mechanism of tumor killing we repeated these experiments using complement inhibitor sCR1 or heat inactivated serum and noted restoration of viability to 95%.

Furthermore, *Griffonia simplicifolia* derived IB_4 FITC labeled isolectin specific for αgal epitopes stained MC38 cells and HE7αgal1 transduced tumors equally demonstrating cell surface expression of αgal in tranduced cells. Parental and mock transduced tumors were negative upon IB_4 isolectin staining. Based on these results we are further exploring the use of αGT in syngeneic αGT knock-out tumor models in an effort to understand the mechanism of tumor destruction *in vivo*. This work represents for the first time delivery of αGT via a herpes viral vector to tumors cells and subsequent direct tumor destruction *in vitro* using human serum.

Evidence for a protective immune stimulation from α (1,3) galactosyl epitopes in mice Unfer, R.C., Hellrung, D., and Link C.J Human Gene Therapy Research Institute, John Stoddard Cancer Center, 1415 Woodland Ave. Suite 218 Des Moines, IA 50309

The α (1,3) galactosyl epitope (αgal) is recognized by the human immune system during complement-mediated hyperacute xenograft rejection. The future goal of *in vivo* delivery of the αgal gene into αgal-negative tumor cells, it's expression, and their subsequent killing is dependent upon demonstration of a protective immunity generated by exposure to αgal. Previous *in vitro* work by our Institute and others has demonstrated cytolysis of αgal transduced tumor cells by human serum. We designed two experiments to determine whether immune priming with αgal would provide any evidence for protection from tumor cell growth. In the first part of this study, C57BL/6 α (1,3) galactosyltransferase knockout mice (αgal-negative) immunized with αgal-rich rabbit RBC, and two non-immunized control groups of C57BL/6 α (1,3) KO mice, and wild-type C57BL/6 mice were implanted with dilutions of αgal-positive MC 38 colonic adenocarcinoma cells. Mice were monitored for tumor growth and morbidity. Results indicate a protective immunity was generated by prior exposure to the αgal epitope in immunized C57BL/6 α (1,3) KO mice signified by lower morbidity and increase survival. In the second part of this study, C57BL/6 α (1,3) galactosyltransferase KO mice were immunized with rabbit RBC, implanted with 10^5 cells of the αgal-negative B16.F10 tumor cell line and divided into three groups. Group A received only RBC and tumor cells, while groups B and C were implanted with 10^7 αgal-positive vector-producing cells (VPC). Group 3 mice were also treated with GCV (10μg/ml). Increased survival was observed among group C mice indicating a protective effect from both exposure to αgal-positive VPC and GCV. These data suggest that innate immunity in humans might be directed against α (1,3) galactosyltransferase expressing tumors.

Generation of a recombinant adenovirus carrying the gene for α(1,3) galactosyl transferase, for use as a suicide gene in cancer immunotherapy.

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The α(1,3) galactosyl transferase enzyme (αGT) is expressed in most mammals but not in humans. αGT adds an α(1,3) linkage of galactose onto glycoproteins or glycolipids, and is a key determinant of tissue and organ xenograft rejection. The α(1,3) galactosyl epitope (αgal) is recognized by innate human immunity resulting in antibody dependent complement-mediated hyperacute xenograft rejection. In human cells, αGT is present as a pseudogene due to two single base deletions. Our previous in vitro work has demonstrated that human tumor cells transduced with a retroviral vector expressing the αGT gene are destroyed by anti-αgal antibody and complement. Additionally, using the MC38 (αgal+) colon adenocarcinoma cell line and αGT knockout mice that have been immunized with rabbit red blood cells (αgal+), we have also demonstrated in vivo that a strong immune response to agal will provide protection from tumor challenge. To achieve a high level of gene transfer, we have cloned the αGT gene into an adenovirus shuttle-vector, and used this to generate a recombinant adenovirus that carries the αGT gene. IGROV human ovarian cancer cells transduced with an MOI of 100 show high levels of expression of the agal epitope when stained with FITC-labeled IB4 lectin. This vector is being evaluated in a variety of human tumor cells, and an in vivo model is under development. The αGT gene may be an ideal suicide gene for immunotherapy of solid tumors using present gene delivery technology. The expression of this transgene by tumor cells and resulting modification of both cellular glycoproteins and glycolipids may provide multiple targets for the immune system, and should result in tumor destruction that is not dependent upon further pharmacological intervention.

Suicide gene expression by an HSV amplicon vector using a complementation system of an IE3-deficient Herpesvirus with a recombinant Adenovirus expressing the HSV-ICP4 gene in co-infected cells.

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The pHE700 Herpes Simplex amplicon system provides an efficient method to generate a vector that can deliver therapeutic genes to target cells while maintaining a replication-incompetent viral vector for the highest safety (Gene Therapy 1997 4:1132). We have previously demonstrated that this method can be used to deliver the suicide gene, α(1,3)galactosyltransferase (αGT), to tumor cells (Adv Exp Med Biol. 2000 465:217). Exposure of amplicon-vector infected cells expressing the αGal epitope on cell surface glycoproteins to human serum, results in a complement-meditated lysis of tumor cells. We are exploring alternative methods of generating a replication-defective amplicon vector that would maintain the ability to deliver a therapeutic gene, while still preserving safety. ICP4 is the major transcriptional regulatory protein of HSV-1, that supports replication of IE3-defective herpes helper virus particles. Replication-defective recombinant adenoviruses have been shown to infect and transfer genes in a wide variety of cell types. We have used a recombinant adenovirus (Ad-ICP4) carrying the HSV-ICP4 gene to infect the VA13 cell line (SV40 transformed human W138 fibroblast) previously selected to carry the pHE700αGT amplicon as an episome. Super-infection of these cells with a replication-incompetent herpes helper virus (CgalΔ3), yields infectious packaged virus particles that contain the αGT gene. Viral supernatant derived from the complementation was added to fresh VA13 cells, and αGT expression was demonstrated by FITC-IB4 lectin-specific labeling of the αGal epitope expressed on cell surface glycoproteins. Experiments are proceeding with the SKBR-3 and T47D breast cancer cell lines, to demonstrate that complementation of defective herpes helper virus with recombinant Ad-ICP4 and resulting αGT expression will sensitize tumor cells to destruction by human serum.

Immunity to the α(1,3)Galactosyl Epitope Provides Protection in Mice Challenged with Colon Cancer Cells Expressing α(1,3)Galactosyl-transferase: A Novel Suicide Gene for Cancer Gene Therapy

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[2] The abbreviations used are: αGal, α(1,3)Galactosyl epitope; αGT, α(1,3)Galactosyltransferase enzyme; αGT KO, α(1,3)Galactosyltransferase Knockout

[3] Keywords: Gene Therapy; α(1,3)Galactosyltransferase; Tumor Vaccine; Suicide Gene; MC38 Colon Carcinoma

ABSTRACT

Human immunity to α(1,3)Galactosyl epitopes (αGal) may provide the means for a successful cancer gene therapy that utilizes the immune system to identify, and destroy tumor cells expressing the suicide gene α(1,3)Galactosyltransferase (αGT). Innate antibody specific for cell surface αGal constitutes a high percentage of circulating IgG and IgM immunoglobulins in humans, and is the basis for complement-mediated hyperacute xenograft rejection, and antibody-dependent cell-mediated cytotoxicity (ADCC). In humans, the gene for αGT is mutated and cells do not express the αGal moiety. We hypothesized that human tumor cells induced to express the αGal epitope would be killed by the hosts' innate immunity. Previous *in vitro* work by our group has demonstrated complement-mediated lysis of αGal-transduced human tumor cells in culture by human serum. To induce antibodies to αGal in this *in vivo* study, α(1,3)Galactosyltransferase knockout mice were employed to determine if immunization with to αGal could provide protection from challenge with αGal-expressing murine MC38 colon cancer cells. Knockout mice were immunized either a single time, or twice, with rabbit RBC. Antibody titers to αGal measured by indirect ELISA, were significantly higher in mice immunized twice, and approached the titers observed in human serum. Anti-αGal antibodies were predominantly of the IgG1 and IgG3 subtype. Immunized knockout mice were challenged intraperitoneally with varying doses of αGal^+ MC38 colon carcinoma cells. Non-immunized control groups consisting of αGT knockout mice, and wild-type C57BL/6 mice were challenged as well with MC38 cells. Immunized mice survived, and exhibited slower tumor development in comparison to

non-immunized knockout and control mice. This study demonstrates *in vivo,* the protective benefit of an immune response to the αGal epitope. Our results provide a basis to pursue further development of this cancer gene therapy strategy.

INTRODUCTION

Cancer gene therapy offers a potential replacement or augmentation of traditional cancer treatments, which utilize invasive or toxic protocols. Suicide genes that encode an enzyme that activates a prodrug into a toxic molecule, or genes that induce apoptosis, have been or are currently being tested in clinical trials for their efficacy in cancer therapy (1-3). Gene therapy vaccine technology is under development for several malignancies. Melanoma has been the favored target because it is a very immunogenic tumor. However, the more common and important forms of cancer have minimal or no immunogenicity. We are developing a novel approach to cancer gene therapy that utilizes a patient's innate immunity against xenoantigens, to identify and destroy tumor cells. The key aspect of this work is to determine if colon cancer cells that express a xenoantigen can be rejected by an immune response to the antigen. Colon cancer is the third-most common form of cancer, and third leading cause of death (4), and provides a good target for a successful cancer gene therapy.

Humans possess specific humoral immunity to the xenotransplant antigen, α(1,3)Galactosyltransferase (αGal). Human cells do not express αGal due to a two base frame-shift mutation (5). Evidence suggests that high titer natural antibody to αGal is produced in humans as a result of continuous antigenic stimulation by gastrointestinal bacteria (5-7). Clonal B-cell analyses estimated that approximately 1% of circulating B cells produce anti-αGal antibody (8). The α(1,3)Galactosyltransferase enzyme (αGT) catalyzes the transfer of galactose from uridine diphosphate galactose (UDP-Gal) to the N-acetyl-lactosamine acceptors on carbohydrate side chains of glycoproteins and glycolipids, to create the αGal moiety. The anti-αGal immune response is responsible

for initiating hyperacute rejection of vascularized xenotransplants, a severe immunological reaction observed in primates. When αGal and specific antibody form immune complexes, complement is activated via the classical pathway (9-13).

Our interest in αGal-mediated destruction of tumor cells was inspired by studies describing lysis of murine retroviral vector producer cells (VPC) following exposure to human peritoneal fluid. VPC have been used for *in vivo* gene delivery in several cancer gene therapy studies (14, 15). Our laboratory and others have demonstrated that antibody and complement in human serum binds αGal within thirty minutes of exposure, and induces complement-mediated lysis of VPC and the viral vectors they produce (16-20). Additionally, Collins et al. showed that human fibroblast cells expressing porcine αGT, were destroyed by antibody and complement (21). To test whether this gene could be used to induce destruction of tumor cells, a truncated version of the murine αGT enzyme was cloned into a retroviral vector backbone and used to transduce human A375 melanoma cells (22). During *in vitro* experiments, greater than 90% of transduced A375 cells expressing αGal were killed following exposure to human serum. αGal expressing A375 cells were treated for thirty minutes with human serum and then injected *in vivo* into athymic nude mice. All experimental mice remained tumor-free while control groups developed tumors (22). Lysis of αGal-expressing murine cells by human serum, can be blocked by the addition of complement inhibitors (heparin, enoxaparin), or sCR1 (soluble complement receptor 1) (20). These data demonstrate the key role that complement has in destruction of targets expressing the αGal xenoantigen.

Transgenic knockout mice that lack the αGT gene (αGT KO), have been produced (23, 24). These mice provide an ideal small animal model to study the *in vivo*

immune response against αGal epitopes, because cells from αGT KO mice do not express detectable αGal epitopes on their surface. αGT KO mice can produce low titers of natural anti-αGal antibody just above background levels, possibly from bacterial stimulation (25, 26). These mice can be effectively immunized with rabbit red blood cell (RRBC) membranes, resulting in the production of anti-αGal antibody with titers and specificity similar to those observed in humans (27). In this report we present *in vivo* data that shows clear protective benefits of an anti-αGal immune response, when RRBC-immunized αGT KO mice are challenged with αGal^+ tumor cells. These findings have implications for generation of a system to deliver the αGal suicide gene to human tumor cells, and making them susceptible to destruction by natural human immunity to αGal.

MATERIALS AND METHODS

Cells and Media. MC38 colon carcinoma cells are syngeneic for C57BL/6 mice, and express the αGal antigen on their cellular surface. B16.BL6-2 melanoma cells, a metastatic non-immunogenic derivative of the B16.F10 cell line, are also syngeneic for C57BL/6 mice, and are αGal-negative. All cells were maintained at 37°C in a 5% CO_2 incubator. The growth medium consisted of Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen-Life Technologies, Carlsbad, CA) supplemented with 10% FBS (D-10) (Invitrogen-Life Technologies).

Animal Model. Knockout mice for αGT (αGT KO) were received for establishing a breeding colony from Dr. J.B. Lowe of the University of Michigan (23). C57BL/6 mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN), and were used as control mice during tumor implantation studies. All animals were cared for under IACUC approved protocol and housed in a contained facility.

Lectin Staining for αGal Epitopes. MC38 and B16.BL6-2 cells were seeded into 30mm dishes in D-10, incubated at 37°C in 5% CO_2, and grown to confluent monolayers. Cell monolayers were washed twice with Hank's Balanced Salt solution (HBSS), and incubated for 15 min at room temperature with a 1:50 dilution in Opti-MEM (Invitrogen-Life Technologies, Carlsbad, CA) of Fluorescein isothiocyanate (FITC)-labeled Griffonia simplicifolia isolectin B4 (IB4), (Vector Laboratories Inc., Burlingame, CA). This lectin has previously been shown to bind specifically to αGal epitopes (28, 29). The lectin

solution was removed, monolayers were washed twice with HBSS, and fresh Opti-MEM was added to cells. Monolayers were observed for lectin binding using a Nikon Diaphot 300 Fluorescent microscope (Nikon Inc. Melville, NY), and photographed using Fuji 1600 Provia color film (Fuji Photo Film Co, Tokyo, Japan).

Complement-Mediated Cell Death. MC38 cells in culture were trypsinized for 2-3 min at 37°C. The trypsin was inactivated with complete culture media (D-10), and cells were collected by centrifugation at 3000 RPM for 5 min at 4°C. Cells were suspended in 200 µl of one of three possible treatment solutions. (50% DMEM and 15% FBS (D-15) in Opti-MEM, 50% fresh human serum in Opti-MEM, or 50% heat inactivated human serum in Opti-MEM). And cells were incubated at 37°C for 1 hr. Treated cells were collected, resuspended in 100 µl of FITC-labeled IB4 lectin (diluted 1:100 in Opti-MEM), and incubated at room temperature for 10 min. One hundred µl of a propidium iodide solution (PI) (25 µg/ml) diluted in HBSS was added to the cells, and incubated for 5 min at room temperature. Cells were collected, resuspended in Opti-MEM, and analyzed by flow cytometry (Coulter Epics Altra Flow Cytometer, Miami, FL).

αGal Antigen Immunization. Eight to twelve week old αGT KO mice were used in this study and cared for under an approved animal protocol using AAALAC guidelines. Mice were immunized intraperitoneally, with 10^7 αGal^+ female NZW rabbit red blood cells (RRBC) (Cocalico Biologicals Inc, Reamstown, PA) suspended in 100 µl HBSS. Immunizations were given either a single time 14 days prior to MC38 and B16.BL6-2 tumor cell challenge, or two times at 28 days and 14 days prior to MC38 tumor cell

challenge. Control αGT KO, and control C57BL/6 mice were mock immunized with 100 µl of HBSS intraperitoneally.

Antibody Titration and Subtyping. Antibody specific for the αGal epitope was detected and end-point titrated using an indirect ELISA. αGal antigen (αGal-BSA, V-Labs Inc. Covington, LA) was diluted to 5 µg/ml in carbonate buffer (pH 9.5), and coated onto PVC ELISA plates (Falcon 3912, Becton Dickinson Labware, Franklin Lakes, NJ) overnight at 37°C in a humidified chamber. Non-specific binding sites in assay wells were blocked for 2 hr with a solution of 1% BSA (Fraction V, Sigma) in carbonate buffer. Two-fold serial dilutions of primary sera were made in wash buffer (1X PBS pH 7.4, 0.05% Tween-20), added to antigen coated wells, and incubated for 1 hr at 37°C. Wells were washed 5 times, and a secondary antibody, HRP-labeled goat anti-mouse IgG H+L diluted 1:5000 in wash buffer (Pierce Chemical Co. Rockford, IL) was added to assay wells, and incubated 1 hr at room temperature. Wells were washed 5 times, and 100µl of 3,3',5',5-tetramethylbenzidine (TMB) liquid substrate (Sigma) was added. Following a 15 min incubation at room temperature, the substrate reaction was stopped with 0.5 N H_2SO_4, and the absorbance at 450 nm for each well was determined using a Molecular Dynamics SpectraMax 250 Plate reader (Sunnyvale, CA).

The murine immune response to αGal over time, was measured by collecting blood from the saphenous vein of mice, on various days (day 1 through day 26) following immunization. Serum was diluted 1:100 in wash buffer prior to detection by ELISA as before. Anti-αGal antibody class and subtype was determined from serum collected 14 days following the last RRBC immununization, using a Zymed MonoAb ID ELISA kit

(Zymed Laboratories Inc, San Francisco, CA). αGal-BSA antigen was coated onto PVC ELISA plate wells as before, and non-specific binding sites were blocked using 1% BSA. Diluted primary sera was added and incubated with antigen. After washing, secondary rabbit anti-mouse isotype HRP-labeled antibodies and TMB substrate were used to identify the murine isotype.

Tumor Cell Challenge. In the first experiment, 15 αGT KO mice were immunized intraperitoneally a single time with 10^7 RRBC in 100 μl of HBSS 14 days prior to tumor cell challenge. As experimental controls, 15 αGT KO mice, and 15 syngeneic C57BL/6 mice were mock-immunized with HBSS. In a blinded experiment, all mice were divided into three sets, with each set comprised of five immunized αGT KO mice, and five mice each of the two control groups. Each set of mice was challenged intraperitoneally with a different dilution of MC38 colon carcinoma cells suspended in Plasma-Lite (Baxter Healthcare Corp. Deerfield, IL). Mice in Set A were challenged with 2.5×10^4 MC38 cells. Mice in Sets B and C were challenged with 5.0×10^4 and 1.0×10^5 MC38 cells respectively. Separately, eight αGT KO mice were immunized a single time as before with 10^7 RRBC in HBSS 14 days prior to intraperitoneal challenge with 1.0×10^5 B16.BL6-2 melanoma cells suspended in Plasma-Lite. A second experiment was designed in which 15 αGT KO mice were immunized twice with 10^7 RRBC, 28 days and 14 days prior to MC38 tumor cell challenge. 15 αGT KO mice, and 15 syngeneic C57BL/6 mice were mock-immunized with HBSS and served as experimental controls. All RRBC-immunized and mock-immunized control mice were challenged

intraperitoneally with 2.5 x 10^4 MC38 colon carcinoma cells. In all experiments, mice were observed daily for animal morbidity and palpated for tumor growth.

RESULTS

FITC-Lectin Staining. MC38 colon carcinoma and B16.BL6-2 melanoma cells were incubated with a solution of FITC-labeled IB4 lectin to identify the differences between the two cell lines in expression of the αGal epitope. FITC staining is prominent along the outer surface membrane of cultured MC38 cells (Fig. 1) since this cell line has a functional αGT gene and expresses the surface αGal moiety that is detected by IB4 lectin binding. B16.BL6-2 melanoma cells do not express the αGal moiety, because they lack a functional αGT gene, and are not stained with IB4 lectin-FITC.

Complement-Mediated Cell Death. αGal-positive MC38 cells, were incubated with culture medium containing no human serum, 50% normal human serum, or 50% heat inactivated human serum. Flow cytometry of FITC-lectin stained cells provided total cell counts. And propidium iodide uptake by dead cells as a percentage of total cell numbers, was used to measure cell death following incubation with the test media. Flow cytometry of cells treated with the three test media, demonstrated killing of 98% of αGal-positive MC38 cells by media containing human serum with active complement (Fig. 2). Cells that were incubated with media containing heat-inactivated human serum, exhibited a 30% cell death by propidium iodide uptake, while background uptake by untreated cells was 22%.

Antibody Titers and Subtyping. Antibody titers to the αGal epitope from immunized αGT KO mice and mock-immunized control αGT KO and C57BL/6 mice, were

determined by indirect ELISA. Figure 3 shows the results of antibody titration in serum from mice that were immunized either a single time 14 days prior to serum collection (Fig. 3A), or mice that were immunized twice, 28 days and 14 days, prior to serum collection (Fig. 3B). Serum from mock-immunized αGT KO and C57BL/6 mice exhibited an average A_{450} absorbance of less than 0.05 at a 1:50 dilution, and was considered negative. This serum was pooled and used to determine background antibody binding. Immune serums with an A_{450} absorbance of greater than 0.1 above background were considered positive. The average titer of anti-αGal antibody in mice immunized one time with RRBC was 1:1600 (Fig. 3A). Mice that were immunized twice with RRBC had an average titer of 1:8000 (Fig. 3B). The immune response in αGT KO mice to αGal was determined by collecting serum from mice, at various times after RRBC immunization on days 14 and 28. An anamnestic IgG immune response to αGal that peaks at 7 days following the second immunization was observed (Fig. 4A)

Serum samples collected on day 0 from αGT KO mice immunized once on day –14 (14 mice), or twice on days -28 and -14 (8 mice) were assayed for their antibody isotype. An A_{450} absorbency greater than 0.2 for an individual antibody isotype was considered positive. Serum was negative for an antibody isotype and subclass if the A_{450} absorbance was less than 0.2. RRBC immunization stimulated the production of IgM, IgG1 and IgG3 antibodies (Fig. 4B). The predominant subclass was IgG3, which developed in 50% (11 of 22 immunized mice). The IgG1 subclass was observed with higher frequency (36%) then either IgG2a and IgG2b (5% and 13%). All mice immunized regardless of schedule, demonstrated high levels of IgM antibody. And no immunized mice demonstrated any IgA antibody isotype. Interestingly, all immunized

mice developed antibody with κ light chain, and no λ light chain was found in these mice.

Tumor Cell Challenge. Fourteen days following RRBC immunization of αGT KO mice, and mock-immunization of αGT KO and C57BL/6 controls, the mice were divided into three groups and implanted intraperitoneally with different dilutions of MC38 colon carcinoma cells in a blinded experiment. Tumor cell dilutions were made, coded, and randomized prior to injection into mice. All mice were observed daily for tumor development and were euthanized when tumors reached approximately 1200 mm^3, exhibited ascites fluid production, or when the mice were moribund. Survival curves of RRBC-immunized and mock-immunized control mice challenged with the three doses of MC38 tumor cells (2.5×10^4, 5.0×10^4, or 1.0×10^5) are presented (Fig. 5). Immunized αGT KO mice were observed to develop tumors more slowly regardless of the amount of MC38 cells used for challenge. Immunized and mock-immunized mice challenged with the three dilutions of MC38 cells were pooled and analyzed as a group. The percentage of mock-immunized control αGT KO mice (47%) that survived challenge with the three dilutions of MC38 cells was lower, than the percentage of RRBC-immunized αGT KO mice (87%) that were challenged ($P = 0.031$). All three dilutions of MC38 cells were able to rapidly establish tumors in mock-immunized C57BL/6 control mice, and all C57BL/6 control mice were euthanized by 18 days-post tumor cell. In a separate experiment, the survival curve for RRBC-immunized αGT KO mice challenged with 1×10^5 B16.BL6-2 cells demonstrates that immunity to αGal does not protect against

challenge with αGal ˉ melanoma cancer cells (Fig. 6). In this experiment, all 8 immunized αGT KO mice developed tumors rapidly, and were euthanized by day 25.

The challenge experiment was repeated using a single dilution (2.5×10^4) of MC38 tumor cells to challenge αGT KO mice immunized twice with RRBC to increase their antibody titers, and mock-immunized control mice. Following tumor cell challenge, all mice were closely monitored and palpated for tumor growth (Fig. 7). None of the mock-immunized C57BL/6 control mice (0%) survived beyond 17 days post-tumor cell challenge. Only 9 of 15 (60%) mock-immunized αGT KO mice survived beyond 32 days post-challenge. In contrast, all of the immunized αGT KO mice (100%) survived MC38 challenge ($P = 0.0069$), and palpated tumors were smaller and developed much more slowly when compared to mock-immunized αGT KO mice.

DISCUSSION

The role of αGal epitopes in xenograft rejection has been well studied (9-13, 30). Rapid immune recognition of αGal epitopes on the surface of foreign cells results in antibody-mediated cell cytotoxicity and complement mediated lysis of targeted cells. This immunity is a barrier to the use of non-human organs and tissue for transplant purposes. Recent advances in transplant science have resulted in the development of α(1,3)Galactosyltransferase knockout pigs that may overcome immune obstacles to successful xenotransplantation in humans (31). Cancer gene therapy protocols that rely upon murine VPC for delivery of genes, can also be limited by the potent human immune response to αGal epitopes. Both VPC and the vectors they produce are destroyed by anti-αGal immune mechanisms (16-20). Clonal B-cell analysis estimated that approximately 1% of circulating B cells produce anti-αGal antibody (8), and an estimated 1 - 2.4% of circulating IgG, and 3.9 - 8% of IgM are specific anti-αGal antibodies (32, 33). We hypothesize that innate anti-αGal immunity that is disadvantageous for xenograft transplantation and VPC therapies, could be utilized as an advantageous method to induce the destruction of cancer cells. Human cancer cells expressing αGal epitopes on their cell surface would appear as xenoantigens, and induce a strong immune response that destroys them. A murine colon cancer cell line was chosen for this study. Colon cancer is the third-most common form of cancer, and third leading cause of death (4).

The αGT KO mouse is an ideal small animal model to study our hypothesis. The loss of the αGT gene by these mice mimics the evolutionary loss of this gene by ancestral

Old World primates and humans (5). αGT KO mice produce little or no αGal specific antibody (25, 26), but are able to develop an immune response to αGal when immunized with rabbit red blood cells (RRBC). These mice can produce αGal-specific antibody with high titers and specificity in some animals similar to those observed in humans (27). The RRBC immunization protocol we used with the αGT KO mice resulted in high titers of anti-αGal antisera that could be detected by indirect ELISA at a 1:16,000 dilution. Previously, LaTemple et al demonstrated a partially protective immune response when αGT KO mice are vaccinated with αGal-expressing B16-BL6 cells (following stable transfection of αGal-negative B16-BL6 cells with αGT cDNA) and challenged with parental B16-BL6 cells (34).

MC38 murine colon carcinoma cells have a functional αGT enzyme, and express αGal on their cell surface glycoproteins. Figure 1 shows the difference in αGal expression between MC38 colon carcinoma and B16.BL6-2 melanoma cells used in this study. The αGal moiety expressed on the surface of MC38 cells is labeled brightly with IB4 lectin-FITC conjugates, while B16.BL6-2 melanoma cells lack αGal expression on their surface and do not bind the IB4 lectin. A serum exposure assay showed the complement-mediated destruction of αGal-positive MC38 colon carcinoma cells. Cells were incubated with media that contained 50% human serum with active complement, or media that contained 50% heat-inactivated human serum (Fig. 2). Following incubation with the test media, propidium iodide uptake measured by Flow Cytometry was used to estimate the percentage of cells killed. A total of 98% of MC38 cells were killed when exposed to untreated human serum. Control cells that were untreated or treated with

heat-inactivated serum (devoid of active complement) showed propidium iodide uptake of 22% and 30% respectively). Therefore, the presence of active human complement induced dramatically higher killing as expected (22). Takeuchi et al demonstrated similar results using human cells. Transfected cells that express porcine αGT are lysed by human serum with complement (18).

The titer of anti-αGal antibody in immunized αGT KO mice was measured by indirect ELISA. Mice immunized twice with RRBC developed an average titer of 1:8000 (Fig. 3B) that is comparable to measured serum titers from patients receiving VPC treatment (data not shown). Mice that were immunized twice with RRBC developed an anamnestic immune response to the αGal antigen (Fig. 4A). The titer of anti-αGal peaked seven days following the second immunization, and IgG1, IgG3, and IgM are the dominant anti-αGal heavy chain isotypes (Fig 4B).

Tumor challenge studies were designed to determine if immunity to αGal epitopes could provide protection from challenge with αGal-expresing MC38 murine colon carcinoma cells. In the first experiment, αGT KO mice were immunized a single time with 10^7 RRBC and challenged with different dilutions (2.5×10^4, 5.0×10^4, or 1.0×10^5) of MC38 cells. A total of 13 out of 15 αGT KO mice survived tumor challenge, while only 7 of 15 mock-immunized αGT KO mice survived challenge with the same dilutions of MC38 cells ($P = 0.031$). Despite our evidence that mice immunized a single time with RRBC do not develop high titers of anti-αgal antibody, 87% of immunized mice were protected and survived. None of the mock-immunized control C57BL/6 mice survived the tumor challenge, and all were euthanized by day 18. While anti-αGal

antibody could not be detected by ELISA in sera from mock-immunized control mice, others have suggested that αGT KO mice have a low natural titer of anti-αgal antibody (25, 26). This natural antibody in the transgenic knockout mouse may, as hypothesized in humans, arise from stimulation of environmental antigens. The combination of pre-existing low antibody titers and stimulation by αGal-positive MC38 cells, may have allowed 47% of the mock-immunized αGT KO mice to survive challenge with the MC38 cell dilutions. In a separate experiment, αGT KO mice were immunized a single time with 10^7 RRBC and challenged with 1.0×10^5 αGal-negative B16.BL6-2 murine melanoma cells. None of these mice survived beyond day 25 of the tumor challenge (Fig. 6), and results of this experiment provide evidence that anti-αGal antibodies do not provide protection against tumors that do not express the αGT gene. A second MC38 challenge experiment incorporated two RRBC immunizations of αGT KO mice to generate higher titers of anti-Gal antibodies. Immunized and mock-immunized control mice were challenged with 2.5×10^4 of MC38 tumor cells. All 15 immunized αGT KO mice survived MC38 challenge, while only 9 of 15 mock-immunized αGT KO mice survived ($P = 0.0069$ by ANOVA). Again, all mock-immunized C57BL/6 control mice developed tumors rapidly and were euthanized by day 17. Following challenge, mice were observed and palpated daily. Observations included slower development of tumors, and smaller tumors in immunized αGT KO mice compared to mock-immunized mice (data not shown). The survival of 60% of the control mock-immunized αGT KO mice may again be due to a combination of low-titer natural antibody and immune stimulation by the MC38 cells. These data provide a first step towards the development of αGal-based colon cancer vaccines for humans.

Colorectal cancer vaccines are as yet in the experimental stage of development. Potential vaccines based upon 17-1A, 791Tgp, carcinoembryonic antigen (CEA) (35), and the SART3 peptide antigens (36) are currently being tested with marginal but encouraging results. Colon cancer cells expressing a functional αGT gene, will be readily identified by the high percentage of innate circulating human antibodies. This in turn, would lead to hyperacute rejection of such genetically modified cells. Hyperacute rejection of porcine xenotransplants occurs after recognition and binding of multiple glycoprotein epitopes by human serum (13, 37). At least 5 major cell surface glycoprotein groups on porcine cells express αGal epitopes and are detected by human IgM and IgG antibodies, and also bind IB4 lectin (38). Similarly, the MC38 colon carcinoma cells used in this study can also be assumed to present multiple epitopes to the αGT KO murine immune system.

We have developed an *in vivo* animal model to demonstrate that immunity to α(1,3)Galactosyl epitope can protect αGT KO mice against challenge with colon cancer cells that express αGal. These results demonstrate the potential for a cancer gene therapy that utilizes the innate immunity to αGal antibody in humans. Direct gene transfer of αGT to *in vivo* tumors will be the key the next step in determining if αGT gene therapy for colon cancer will be successful. Gene delivery to tumor cells and expression of the αGT gene will present multiple targets for the immune system. Since both opsonization and complement fixation are dependent upon epitope density, the potential for a protective immune response directed against human tumor cells that express αGal is great.

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Fig. 1. Visualization of αGal expression in MC38 Colon Carcinoma cells and lack of expression in αGal-negative B16.BL6-2 Melanoma cells using FITC-IB4 lectin staining. Cells were incubated with a 1:50 dilution of FITC-IB4 lectin in Opti-MEM at room temperature for 15 minutes. Panels A and B show MC38 cells, and panel C and D show B16.BL6-2 cells. FITC-lectin staining of the αGal moiety in the outer cell surface membrane demonstrates the activity of the αGT gene in MC38 cells, and lack of gene expression in B16.BL6-2 cells. Panels A and C were photographed using a Nikon DM505 FITC cube, and panels B and D were photographed under brightfield light using the same FITC cube.

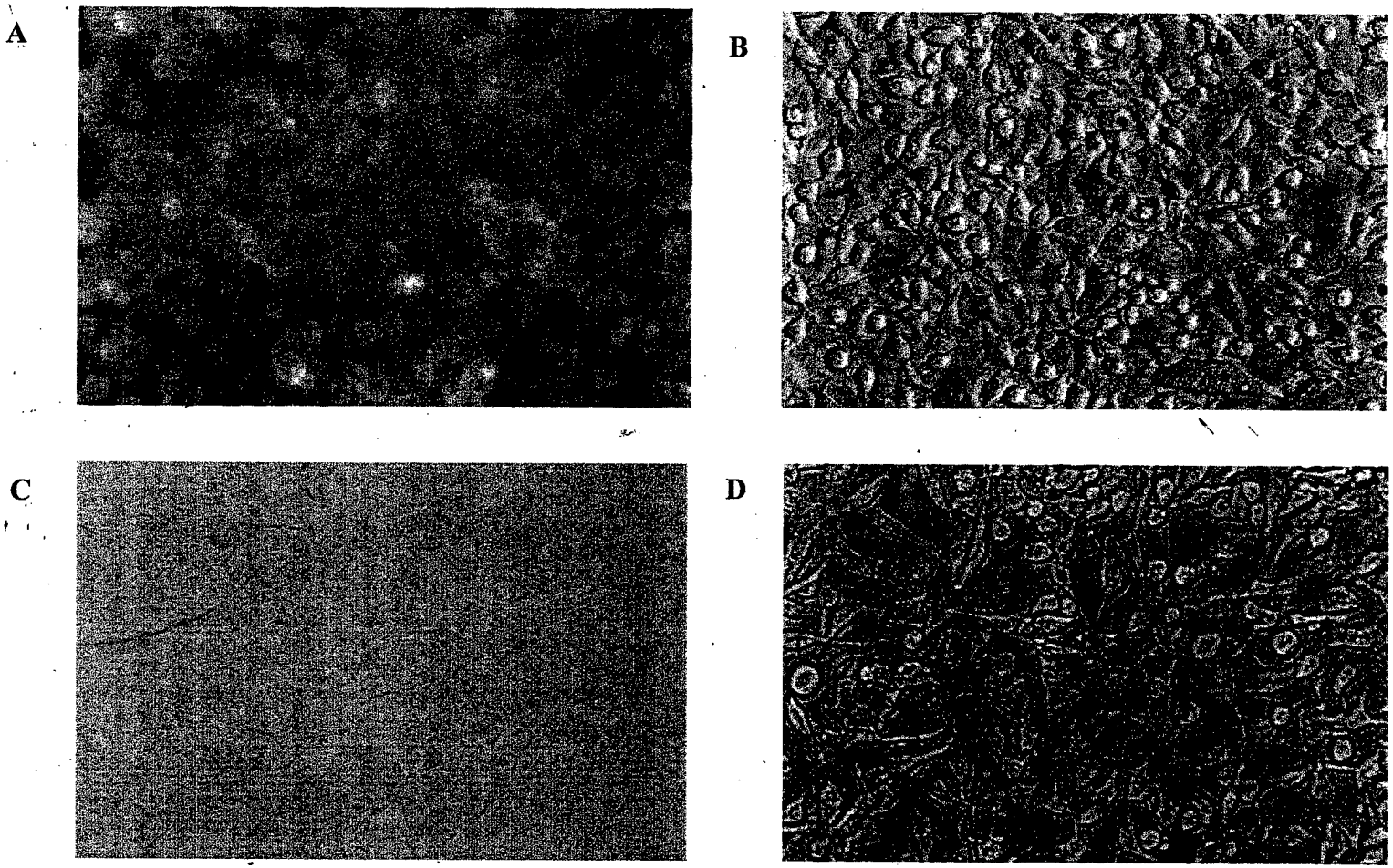

A
B
C
D

Fig. 2. αGal-positive MC38 colon carcinoma cells are killed when exposed to serum containing active human complement. MC38 cells were stained with FITC-IB4 lectin, and suspended in test media containing no human serum, 50% human serum, and 50% heat-inactivated human serum. Following 1 h incubation at 37°C, cells were incubated with a suspension of propidium iodide for 5 minutes at room temperature. Flow cytometry demonstrated that only serum containing active complement was able to kill αGal expressing MC38 cells.

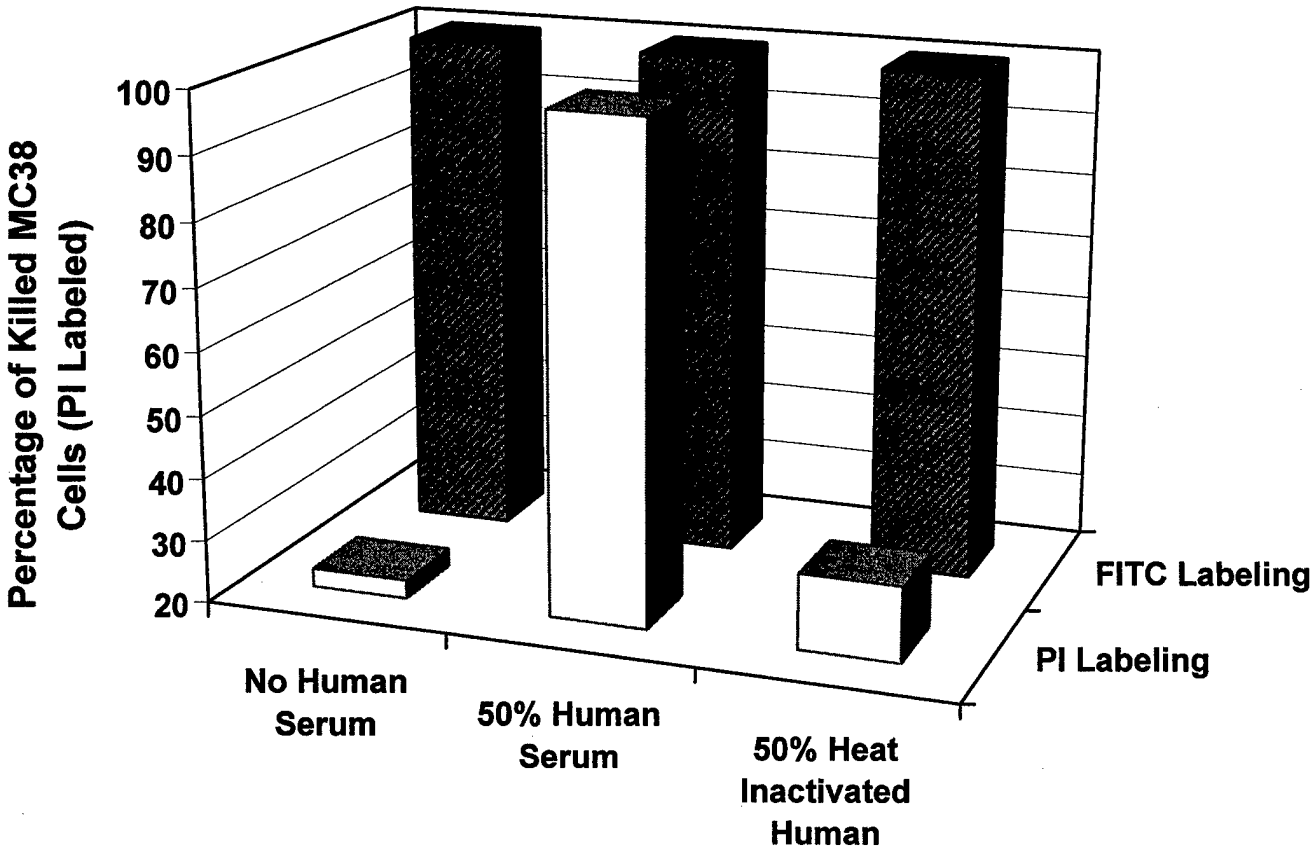
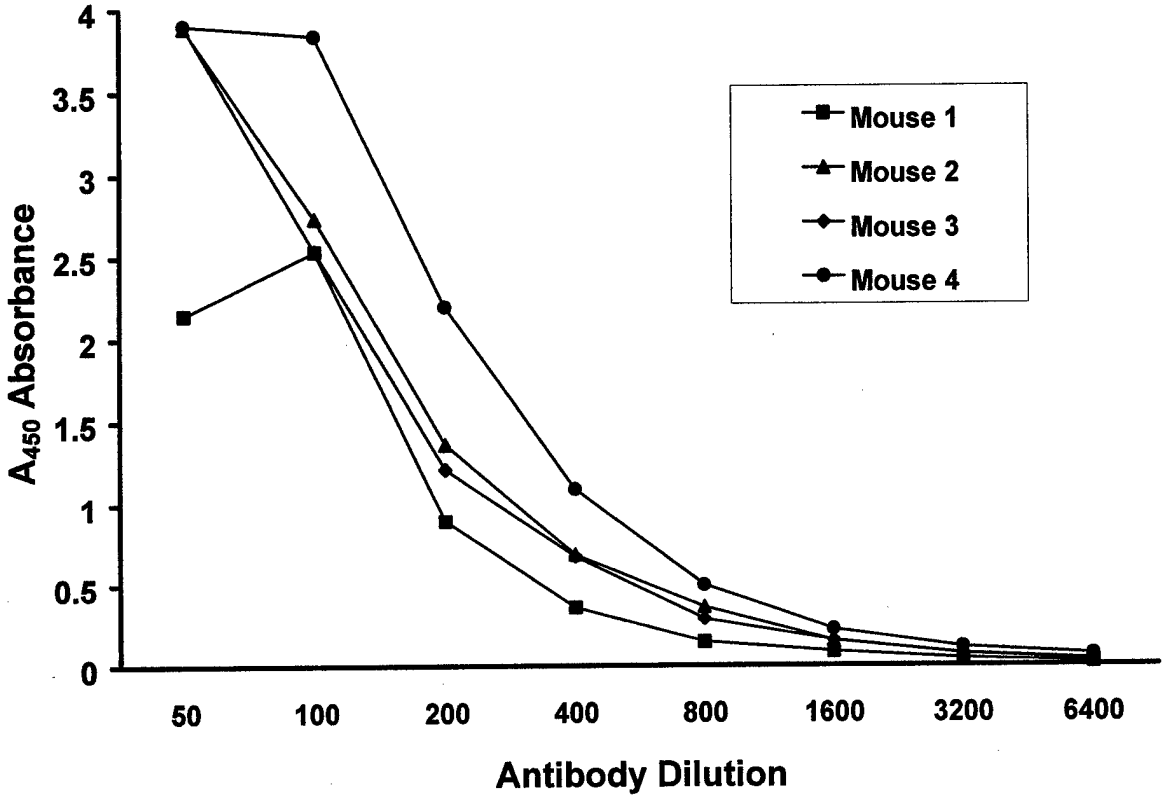
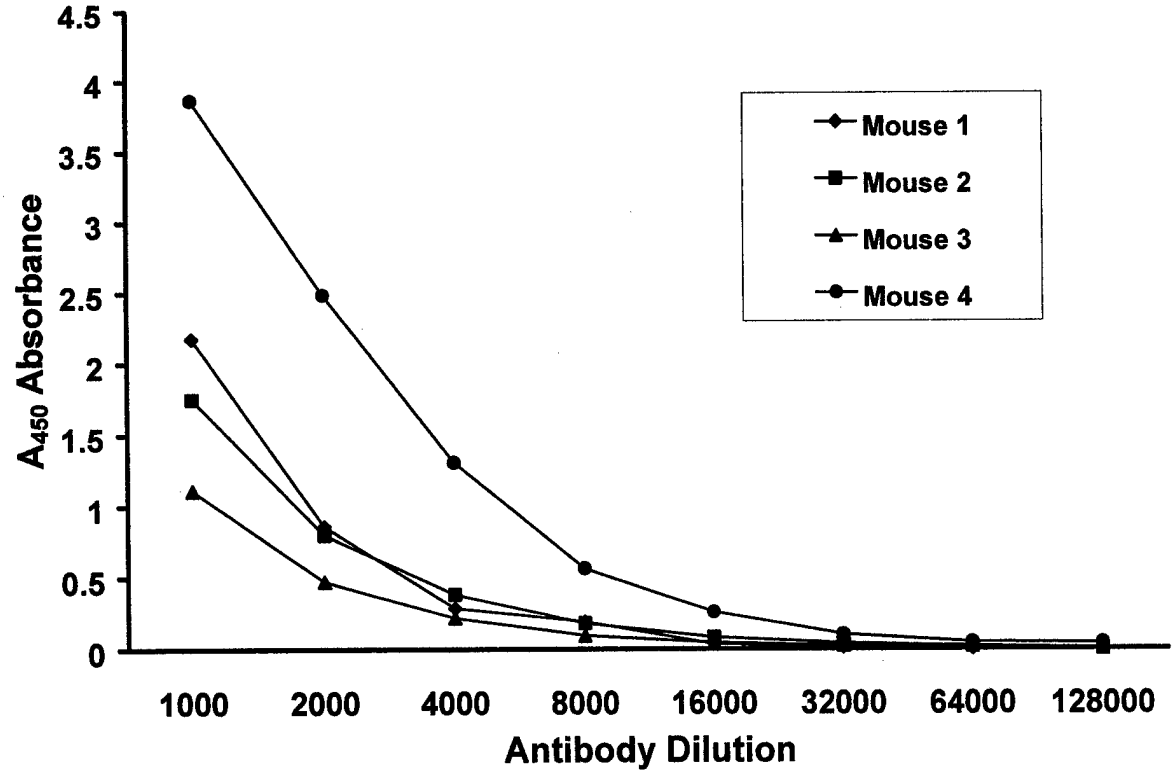

Percentage of Killed MC38 Cells (PI Labeled)
100
90
80
70
60
50
40
30
20
No Human Serum
50% Human Serum
50% Heat Inactivated Human
FITC Labeling
PI Labeling

Fig. 3. High anti-αGal antibody titers are induced in αGT KO mice following immunization with RRBC. An indirect ELISA using an αGal-BSA-conjugate as the antigen was used to titrate serum collected from αGT KO mice that were immunized (A) one time with 10^7 RRBC or (B) two times with a fourteen day interval with 10^7 RRBC. Serum was collected two weeks after completion of immunization. Serum from mock-immunized mice was pooled and used to determine background antibody binding. Immune serums with an A_{450} absorbance of greater than 0.1 above background were considered positive. The average titer of anti-αGal antibody in mice immunized one time with RRBC was 1:1600 (A). Mice that were immunized twice with RRBC had an average titer of 1:8000 (B).

A


4
3.5
3
2.5
2
1.5
1
0.5
0
A450 Absorbance
Mouse 1
Mouse 2
Mouse 3
Mouse 4
50
100
200
400
800
1600
3200
6400
Antibody Dilution

B


4.5
4
3.5
3
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2
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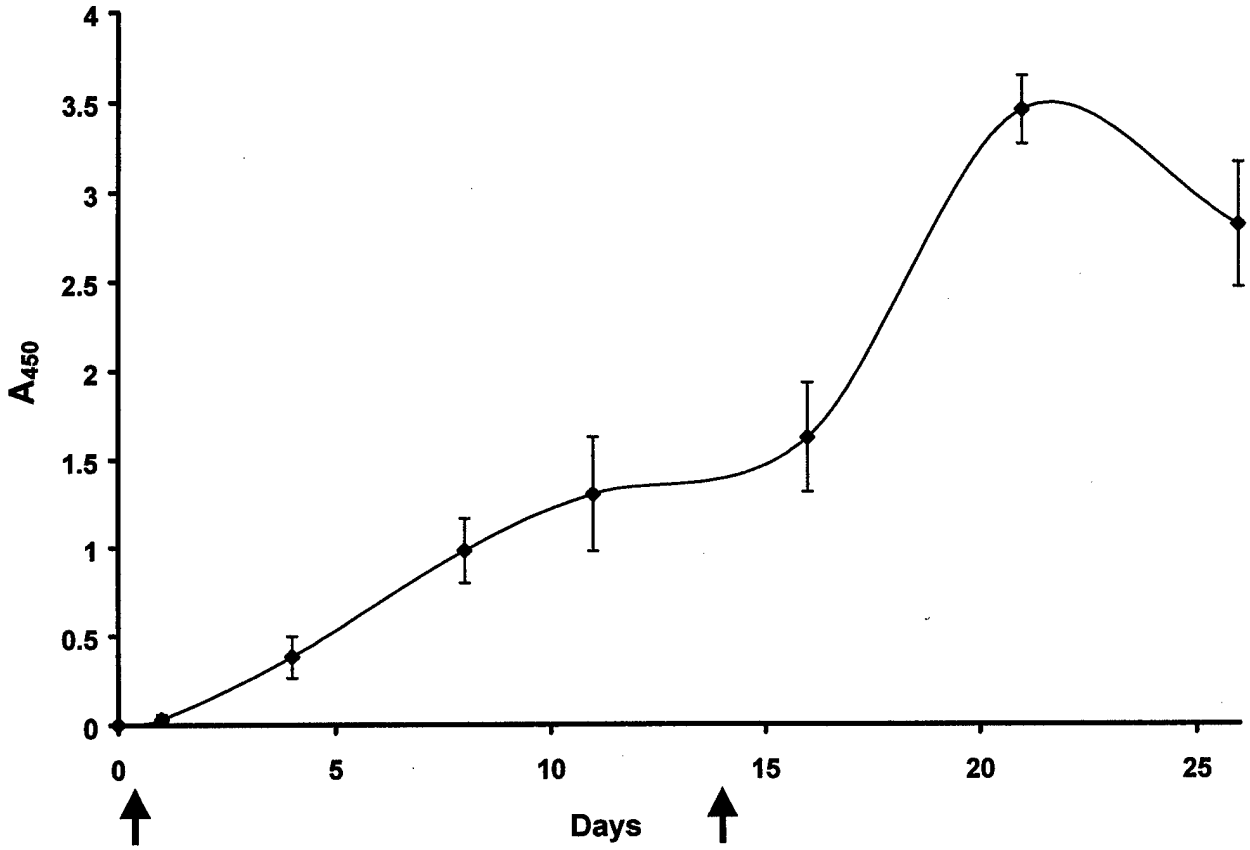
1
0.5
0
A450 Absorbance
Mouse 1
Mouse 2
Mouse 3
Mouse 4
1000
2000
4000
8000
16000
32000
64000
128000
Antibody Dilution

Fig. 4. Immune response to αGal epitopes peaks at 7 days following a second immunization with αGal-positive RRBC. (A) Mice were immunized with 10^7 RRBC on days 0 and 14 (indicated by arrows ↑). Blood was collected from the saphenous vein at various times from 4 days to 26 days after immunizations were initiated. Serum was analyzed using an indirect ELISA to measure the presence of anti-αGal antibodies. An anamnestic response to αGal was observed following the second immunization. (B) Serum from RRBC-immunized mice was used to determine anti-αGal antibody isotypes by indirect ELISA. Mice immunized once or twice all produced IgM heavy chain and κ light chain antibodies. The predominant IgG subtypes for both sets of immunized mice were IgG1 and IgG3.

A



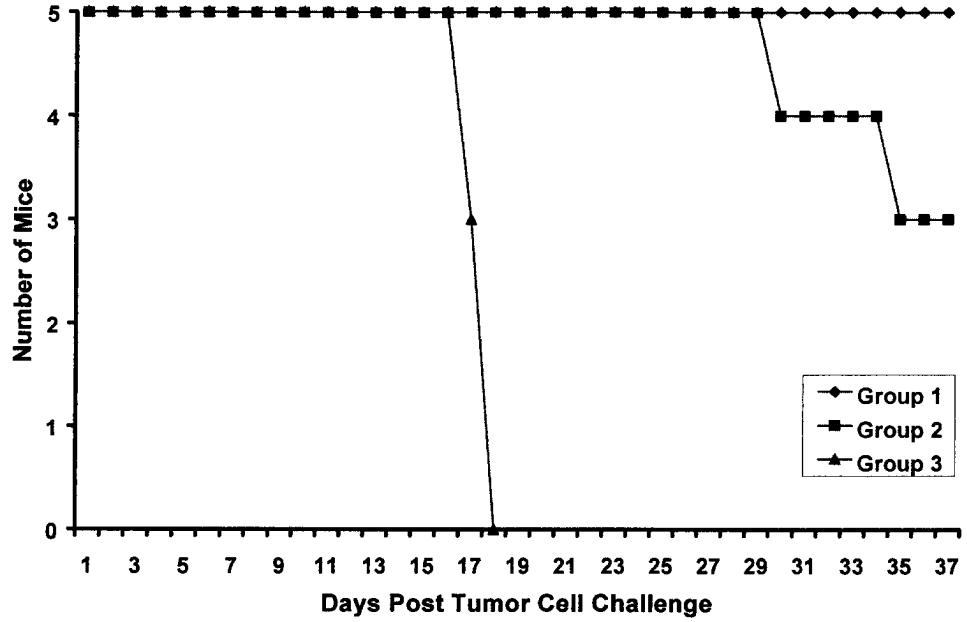
B

Mouse anti-αGal Antibody Isotypes Determined by Indirect ELISA

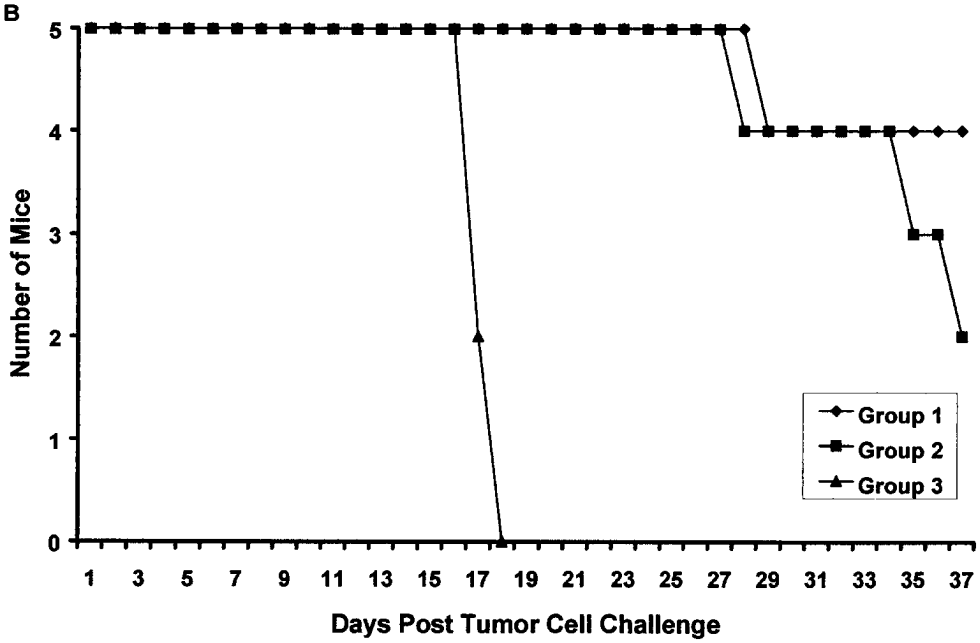
	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM	κ	λ
Mice immunized once (n = 14)	6	1	2	6	0	14	14	0
Mice immunized twice (n = 8)	2	0	1	5	0	8	8	0

Fig. 5. Mice immunized with RRBC showed superior protection to tumor challenge with αGal-positive MC38 colon carcinoma cells, compared to mock-immunized mice. Survival curves for immunized αGT KO mice, and mock-immunized αGT KO mice and C57BL/6 mice following MC38 tumor cell challenge demonstrate that an immune response to αGal epitopes can increase the survival of immunized mice in comparison to mice that are not immunized with αGal. Group 1 consisted of αGT KO mice that were immunized one time with 10^7 RRBC prior to challenge 14 days later with MC38 cells. Group 2 consisted of αGT KO mice that were mock-immunized and challenged 14 days later. Group 3 consisted of C57BL/6 mice that were mock-immunized and challenged 14 days later. Three dilutions of MC38 cells were used to challenge mice (A) 2.5×10^4 MC38 cells, (B) 5.0×10^4 MC38 cells, or (C) 1.0×10^5 MC38 cells. Mock-immunized C57BL/6 mice all died or were euthanized within 18 days. Mock-immunized αGT KO mice showed 40 - 60% survival. Immunized αGT KO mice showed 80 - 100% survival.

A


Number of Mice
0
1
2
3
4
5
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
Days Post Tumor Cell Challenge
Group 1
Group 2
Group 3

B


Number of Mice
0
1
2
3
4
5
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
Days Post Tumor Cell Challenge
Group 1
Group 2
Group 3

C

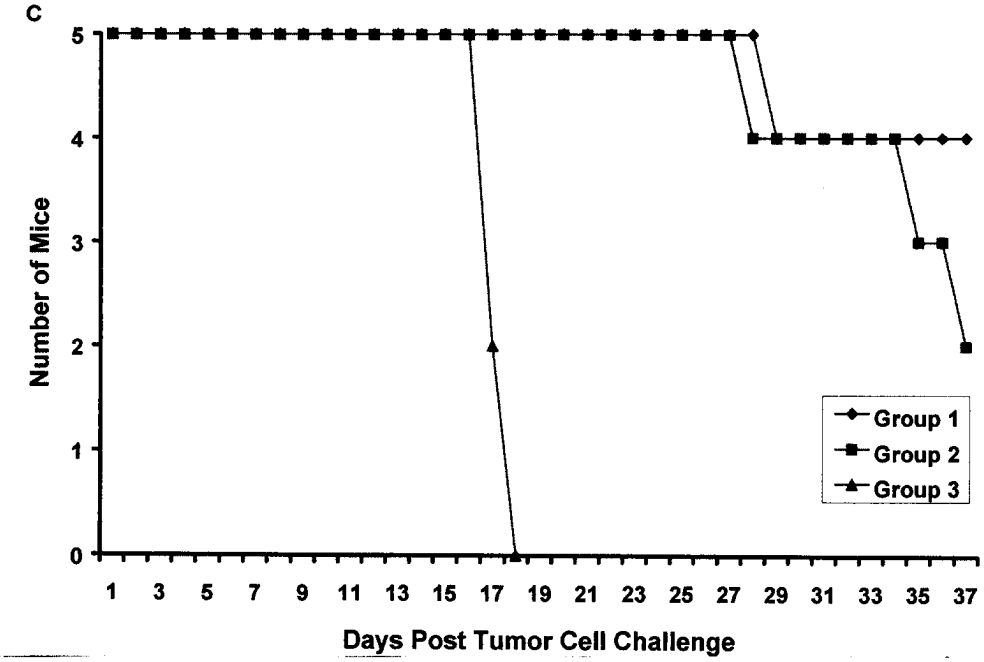
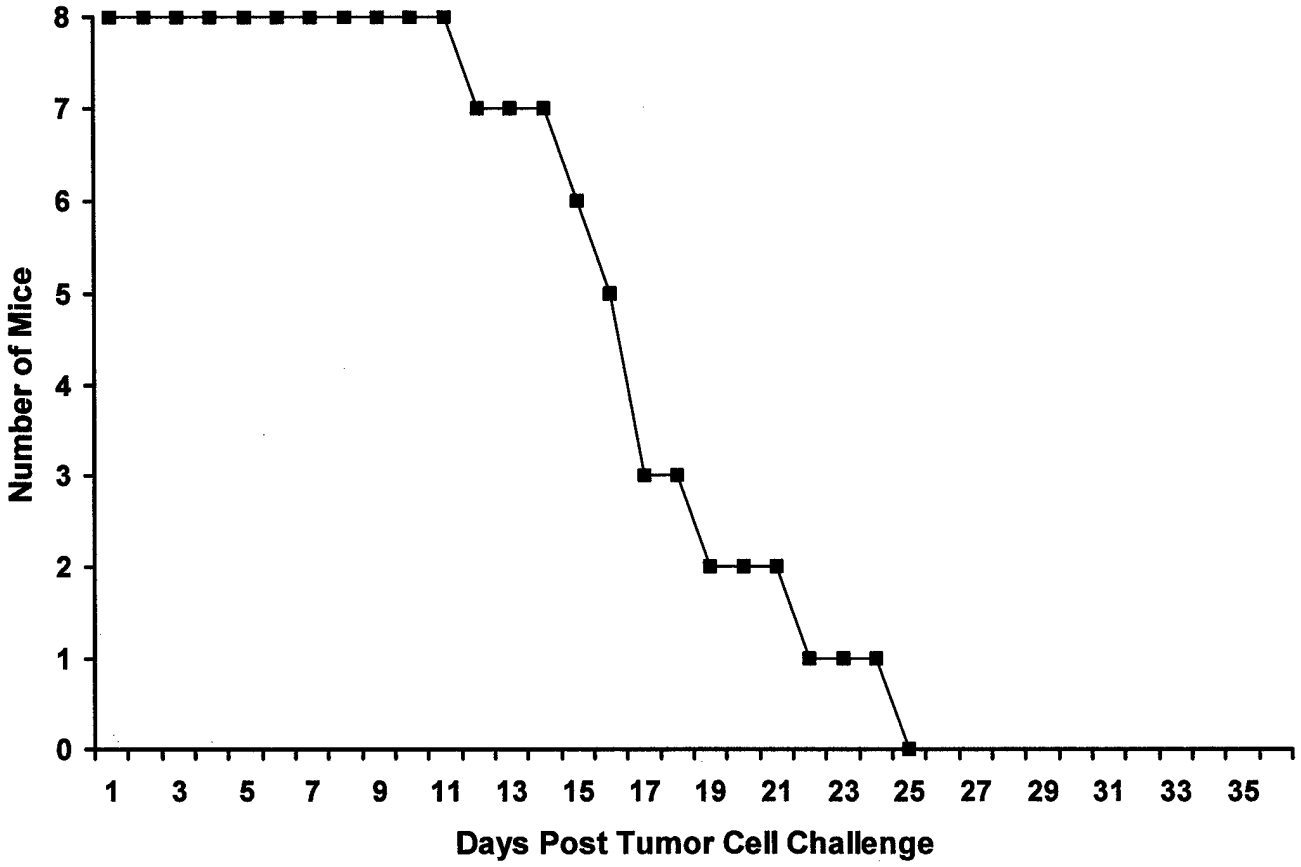

Number of Mice
0
1
2
3
4
5
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
Days Post Tumor Cell Challenge
Group 1
Group 2
Group 3

Fig. 6. Mice immunized with RRBC were not protected following tumor challenge with αGal-negative B16.Bl6-2 melanoma cells. Survival curve for immunized αGT KO mice after challenge with B16.BL6-2 cells shows that an immune response to αGal epitopes does not prevent tumor growth and death. Mice were immunized one time with 10^7 RRBC and challenged 14 days later with 1.0×10^5 B16.BL6-2 melanoma cells. None of the mice survived tumor challenge despite immunization with αGal antigen, because the B16.BL6-2 tumor cell line is a non-αGal expressing cell.


Number of Mice
0
1
2
3
4
5
6
7
8
1
3
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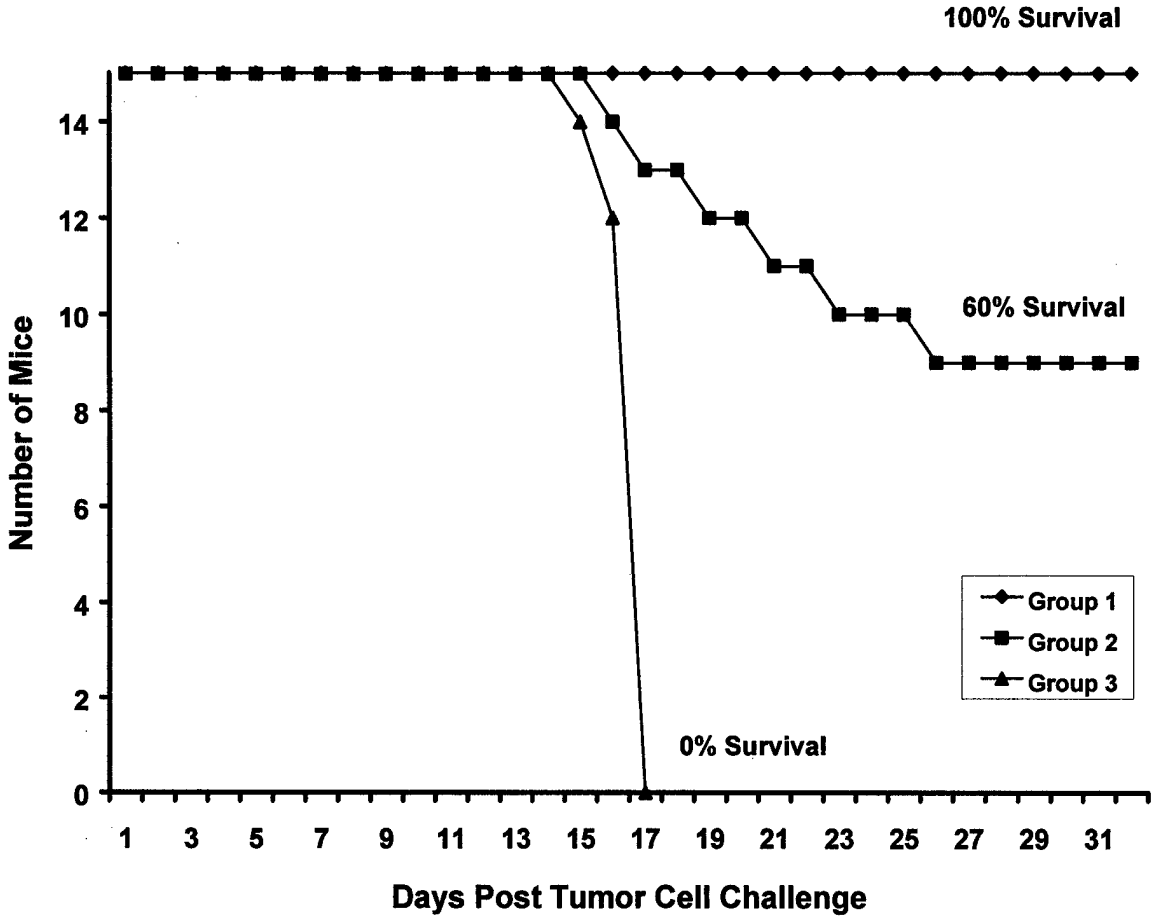
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
Days Post Tumor Cell Challenge

Fig. 7. Mice immunized two times with RRBC showed superior protection to tumor challenge with αGal-positive MC38 colon carcinoma cells, compared to mock-immunized mice. Survival curves for immunized αGT KO mice (Group 1), and mock-immunized αGT KO mice (Group 2) and C57BL/6 mice (Group 3) following MC38 tumor cell challenge. Mice were immunized or mock immunized two times with 10^7 RRBC, 28 days and 14 days prior to tumor cell challenge. All mice were challenged with 2.5×10^4 MC38 cells. Group 1 αGT KO were immunized with RRBC and demonstrated 100% survival to tumor cell challenge ($p<.0069$) in comparison to Group 2 mock-immunized αGT KO mice (60% survival) and Group 3 mock-immunized C57BL/6 mice (0% survival).


100% Survival
60% Survival
0% Survival
Group 1
Group 2
Group 3
Number of Mice
Days Post Tumor Cell Challenge
0
2
4
6
8
10
12
14
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31

October 2, 2002

Breast Cancer Research Program

Department of the Army

Regarding: DAMD17-00-1-0292

Dear Grant Review Member,

We would like to add Dr. Robert Unfer as a co-investigator on the grant. Dr. Unfer has been working closely with the development of adenoviral vectors to deliver the alpha(1,3)galactoslytransferase gene. As well he has been studying the effects of immune protection against alpha(1,3)galactoslytransferase expressing tumors in the alpha(1,3)GT knock out mouse. As such his skills are greatly supportive of our project. Dr. Unfer intends to spend 10% of his time on the project. No budget alterations or additions are requested. I have attached his Curriculum Vitae for your review. Thank you for your attention to this matter.

Sincerely,

Charles J. Link, M.D.

Director

Stoddard Cancer Research Institute

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Robert C. Unfer, Ph.D.	Postdoctoral Fellow

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

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
University of Illinois, Champaign-Urbana	B.S.	1978-1982	Microbiology
Iowa State University	M.S.	1982-1985	Microbiology
Iowa State University	Ph.D.	1986-1991	Microbiology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. 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. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience:

1999- : Postdoctoral Fellow, Human Gene Therapy Research Institute, Des Moines, IA
1996-1999: Research Assistant, University of Colorado Health Sciences Center, Denver, CO
1993-1996: Postdoctoral Fellow, Rush Medical Center, Rush Presbyterian St Lukes Med. Ctr, Chicago, IL
1991-1993: Postdoctoral Scientist, Fort Dodge Laboratories, Fort Dodge, IA
1987-1991: Research Assistant, USDA-ARS, National Animal Disease Center, Ames, IA
1982-1991: Research and Teaching Assistant, Iowa State University, Ames, IA

Professional Organizations:

American Association for Cancer Research
American Society of Gene Therapy
American Society of Clinical Oncology
American Society for Virology

Publications:

1. Wasmoen, T., Kadakia, N.P., **Unfer, R.C.**, Fickbohm, B.L., Cook, C.P., Chu, H-J., Acree, W.M. (1995) Protection of cats from infectious peritonitis by vaccination with a recombinant raccoon Poxvirus expressing the nucleocapsid gene of feline infectious peritonitis virus. In *"Corona-and Related Viruses"* (P.J. Talbot and G.A. Levy, Eds), pp 221-228. Plenum Press, New York
2. Wasmoen, T., Kadakia, N.P., **Unfer, R.C.**, Fickbohm, B.L., Cook, C.P., Chu, H-J., Acree, W.M. (1995) Protection of cats from infectious peritonitis by vaccination with a recombinant raccoon Poxvirus expressing the nucleocapsid gene of feline infectious peritonitis virus. Adv Exp Med Biol. 1995;380:221-8.
3. Tyler, R.C., Fagan, K.A., **Unfer, R.C.**, Gorman, C., McClarrion, M., Bullock, C. and Rodman, D.M (1999) Vascular inflammation inhibits gene transfer to the pulmonary circulation, *in vivo*. *Am J Physiol.* **277**:L1199-204.
4. **Unfer, R.C.**, Hellrung, D. J., Link C. J. Jr. Immunity to the α(1,3)Galactosyl Epitope Provides Protection in Mice Challenged with Colon Cancer Cells Expressing α(1,3)Galactosyltransferase: A Novel Suicide Gene for Cancer Gene Therapy. Manuscript submitted to Cancer Research.
4. **Unfer, R.C.**, Wasmoen, T., Chu, H-J. (2001) Experimental induction of feline infectious peritonitis in cats. Manuscript submitted.

Abstracts:

1. **Unfer, R.C.**, Diaco, R., Nath, S., and Durand, D.P. (1985) Characterization of monoclonal antibodies against the proteins of Newcastle Disease Virus. *Annual. Meeting American Society for Microbiology*, p 298.
2. **Unfer, R.C.**, Diaco, R., and Durand, D.P. (1986) Antigenic characterization of the hemagglutinin-neuraminidase glycoprotein of Newcastle Disease Virus using monoclonal antibodies. *Annual. Meeting American Society for Microbiology*, p 319.
3. **Unfer, R.C.**, Durand, D.P., and Bolin, S.R. (1990) Epitope mapping of the 53kd envelope glycoprotein of bovine viral diarrhea virus. *Annual Meeting CIEDA*, Ames, IA.

4. **Unfer, R.C.**, Bolin, S.R., and Durand, D.P. (1990). Development of a rapid enzyme-linked immunosorbent-assay for the detection of antibody to bovine viral diarrhea virus in fetal calf sera. *Annual Meeting of the North Central Veterinary Laboratory Diagnosticians*, Fargo, ND
5. **Unfer, R.C.**, Bolin, S.R., and Durand, D.P. (1990). Topographic mapping of neutralizing epitopes on the 53kd envelope glycoprotein of bovine viral diarrhea virus. *Annual Meeting of the American Society for Virology,* Salt Lake City, UT
6. **Unfer, R.C.**, Hellrung, D. and Link, Jr C.J. (2000). Evidence for a protective immune stimulation from α(1,3) galactosyl epiitopes in mice. *3rd Annual Meeting of the American Society for Gene Therapy*, Denver, CO
7. Vahanian, N., Mitrofanova, E., Higginbotham, J., Roberson, E., **Unfer, R.C.**, and Link, C.J. (2001). Adenoviral vector delivery of the human sodium/iodide symporter and ^{131}I results in potent in vivo antitumor activity in a solid tumor model. *92nd Annual Meeting of the American Association for Cancer Research, New Orleans LA*
8. **Unfer, R.C.**, Hellrung, D.J., Higginbotham, J., Vahanian, N., Seth, P., and Link, C.J. (2001). Generation of a Recombinant Adenovirus carrying the gene for α(1,3)galactosyl transferase, for use as a suicide gene in cancer immunotherapy. *92nd Annual Meeting of the American Association for Cancer Research, New Orleans LA*
9. **Unfer, R.C.**, Hellrung, D.J., Seregina, T., and Link, C.J. (2001). Use of α(1,3)galactosyltransferase (αGT) as a novel suicide gene for cancer gene therapy. *4th Annual Meeting of the American Society for Gene Therapy, Seattle, WA*
10. Mitrofanova, E., Vahanian, N., **Unfer, R.C.,** Roberson, E., Daniels, W., and Link C.J. (2001). Expression of the rat sodium iodide symporter (NIS) in prostate cancer cells results in the accumulation of ^{131}I in the tumor and inhibition of tumor growth after *in vivo* gene transfer. *Annual Meeting Cancer Gene Therapy, San Diego CA.*
11. **Unfer, R.C.,** Hellrung D.J., Wang, S., Link C.J. (2002). Suicide gene expression by an HSV amplicon vector using a complementation system of an IE3-deficient Herpesvirus with a recombinant Adenovirus expressing the HSV-ICP4 gene in co-infected cells. *93nd Annual Meeting of the American Association for Cancer Research, San Francisco, CA.*
12. Link, C.J., Hellrung, D., Wang, S., Seregina, T., and **Unfer, R.** (2002). A novel therapy for Breast Cancer by inducing hyperacute rejection. *Department of Army, Era of Hope Meeting.*