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13. ABSTRACT ***(Maximum 200 Words)***

This investigation was based on the hypothesis that weakly immunogenic, breast cancer-associated antigens, the products of mutant or dysregulated genes in the malignant cells, will be expressed in a highly immunogenic form by a semiallogeneic IL-2 secreting fibroblast transfected with DNA from breast cancer cells. To investigate this question, we transfected LM mouse fibroblasts ($H2^k$) modified to secrete IL-2 with genomic DNA from breast adenocarcinoma that arose spontaneously in a C3H/He mouse ($H2^k$). To increase their nonspecific immunogenic properties, the fibroblasts were also modified before transfection to express allogeneic MHC determinants ($H\text{-}2K^b$). Afterward, the IL-2 secreting semiallogeneic cells were cotransfected with DNA from the spontaneous breast neoplasm, along with with a plasmid (pHyg) conferring resistance to hygromycin. Pooled colonies of hygromycin resistant cells were then tested in C3H/HeJ mice for their immunogenic properties against the growth of the breast neoplasm. The results indicated that the tumor-bearing mice immunized with the transfected cells survived significantly longer than mice in various control groups and that $CD8^+$ T cells were required for the effectiveness of this type of therapy.

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INTRODUCTION

These studies were undertaken to determine if immunization with semi-allogeneic fibroblasts modified to express IL-2 and transfected with genomic DNA from a breast tumor resulted in anti-breast tumor immunity. It had been previously determined in our laboratory that a similar vaccine constructed with melanoma genomic DNA was effective at generating anti-melanoma immunity (1).We reasoned that genes specifying numerous, unidentified, weakly immunogenic TAAs would be expressed in a highly immunogenic form by the transfected cells, and that immunizations with the transfected cell would result in an immune response directed toward the breast cancer cell.

DNA was isolated from a spontaneously arising breast adenocarcinoma in a C3H/He mouse, spontaneous breast adenocarcinoma 1 (SB1), and was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. To increase their non-specific immunogenic properties and to ensure rejection, before transfection, the fibroblasts were modified to express allogeneic H-2K^b-determinants, and to secrete IL-2. Preliminary results indicated that C3H/HeJ mice were completely susceptible to SB1 growth when as few as 1 x 10^4 cells were injected. A parallel study was carried out in C57BL/6 mice using EO771 breast adenocarcinoma cells. Experiments were performed to determine a) if an anti-breast tumor immune response was generated and b) to define the effector cell populations.

The results indicated that mice immunized with the transfected fibroblast developed immunity toward the breast cancer cells. The first appearance of tumor was delayed and the treated mice survived significantly longer than mice in various control groups, including mice injected with non DNA-transfected LM-IL-2K^b fibroblasts. $CD8^+$ T cells were required for the effectiveness of this type of therapy.

These results raise the possibility that a fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm.

BODY:

LM fibroblasts are of C3H/He mouse origin. They express H-2^k class I determinants and share identity at the MHC with C3H/He mouse origin. To increase the fibroblasts' immunogenic properties, the cells were modified for IL-2 secretion and to express allogeneic (H-$2K^b$) MHC-determinants (semi allogenic cells). After IL-2 secretion and the expression of H-$2K^b$ determinants were confirmed, the modified cells (LM-IL-$2K^b$) were co-transfected with DNA from spontaneous breast cancer arising in individual C3H/He mice. Using C3H/HeJ mice and SB1 (spontaneous mammary adenocarcinoma) as our model, we utilized LM-IL-$2K^b$ cells and transfected them with genomic DNA from SB1 tumor. We found that that tumor growth was delayed in the mice injected with LM-IL-$2K^b$/SB1 and led to prolonged survival of mice with SB1 tumor (Annual report 2000). Another vaccine was prepared using LM-IL-$2K^b$ cells transfected with DNA from a tumor (EO771) which has a histological morphology similar to SB1 yet is from a different host strain (LM-IL-$2K^b$/EO771). The experiment was carried out in C57BL/6 mice injected with E0771 cells and animals treated with LM-IL-$2K^b$/EO771 or LM-IL-$2K^b$/SB1. Although there was some cross reaction in the response against both vaccines a more potent response was elicited by the vaccine transfected with the DNA from the tumor being treated (Annual report 2000).

Involvement of $CD8^+$T cells in the anti-breast tumor immune response

These data suggested that mice immunized with the vaccine constructed from genomic DNA isolated from the tumor, resulted in immunity to the tumor. Since $CD8^+$T cells are the primary effector population responsible for specific, cytolytic anti-tumor immunity, experiments were performed to determine if $CD8^+$T cells were required for the effectiveness of this type of therapy. For this purpose, it was initially determined that a cytolytic immune response could be generated with spleen cells isolated from mice immunized with EO771 and LM-IL-$2K^b$/EO771 cells. As seen in Table I, spleen cells isolated from LM-IL-$2K^b$/EO771 injected mice, resulted in 25±7 % specific lysis of EO771 target cells. Spleen cells isolated from mice injected with LM, LM-IL-$2K^b$, LM-IL-$2K^b$/B16F1 (LM-IL-$2K^b$ cells transfected with DNA from B16 melanoma), or media alone, did not result in significant cytolysis. As a positive control, an allogeneic target cell (LM) was labeled with ^{51}Cr. Spleen cells incubated with Cr-labeled LM cells had significant lysis against LM, providing the mice had been injected with LM cells, ranging between 53 ± 3 to 64 ±15 percent specific lysis. Spleen cells isolated from media injected mice did not lyse either EO771 cells (3.3 ± 1.0) or LM cells (1.8 ± 12).

Table I Cytotoxic responses toward E0771 breast adenocarcinoma cells in C57BL/6 mice injected with a mixture of EO771 cells and LM-IL-2Kb/EO771 cells.

Injected with EO771 cells and	Target	% specific ^{51}Cr-release
LM-IL-2K^b/EO771 cells	EO771	25.0 ± 7
LM cells	EO771	9.0 ± 4
LM-IL-2K^b cells	EO771	3.1 ± 2.0
LM-IL-2K^b/B16F1 cells	EO771	7.0 ± 4.0
Media	EO771	3.3 ± 1.0
EO771 cells and		
LM-IL-2K^b/EO771 cells	LM	59 ± 12
LM cells	LM	64 ± 15
LM-IL-2K^b cells	LM	53 ± 3.0
LM-IL-2K^b/B16F1 cells	LM	57 ± 10
Media	LM	1.8 ± 12

Legend: Mice were injected on day 0 with 5 x 10^3 EO771 cells and 2 x 10^6 LM-IL-2K^b/EO771 cells i.b. An additional injection of 2 x 10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 ul was injected i.p. Injections of 2 x 10^6 LM-IL-2K^b/EO771 cells in a total volume of 200 ul were injected i.b. and i.p. once a week for two additional weeks. One week after the last injection, the spleen cells were isolated, counted and re-stimulated with 2 x 10^6 LM-IL-2K^b/EO771 cells at an effector to stimulator ratio of 30:1. The same procedure was followed for LM, LM-IL-2K^b, and LM-IL-2K^b/B16F1 cells. Media mice were injected with 5 x 10^3 EO771 cells in 200 ul of media i.b., and 200 ul of media alone i.p. The two weekly injections were 200 ul of media injected i.b. and i.p. After 5 days, a Cr-release assay was performed as described in materials and methods. Both Cr-labeled EO771 and LM cells were used at targets for the assay. The effector to target ratio was 100:1.

Together, these data suggested that mice immunized with the vaccine constructed from genomic DNA isolated from the tumor, resulted in systemic immunity to the tumor. However, further experiments were performed to determine if the lysis was mediated by CD8$^+$T cells. Spleen cells isolated from mice immunized with LM-IL-2K^b/EO771 cells were pre-incubated with anti- CD8$^+$T cell antibody. After the addition of complement (to deplete the CD8$^+$T cell population) the remaining cells were incubated with Cr-labeled EO771 cells. The results (TableII) indicate that the percent specific lysis was significantly inhibited in spleen cells obtained from mice immunized with LM-IL-2K^b/EO771 cells (97%). Depleted spleen cells isolated from media, LM-IL-2, or LM-IL-2K^b cell-injected mice did not have significant inhibition of specific lysis to EO771 cells. LM-IL-2K^b/SB-1 cell-injected mice also had significant inhibition (83%). Previous experiments had determined that EO771 cells and SB-1 cells were cross-reactive (Annual report 2000). However, all spleen cells were significantly inhibited after the depletion of CD8$^+$T cells, when reacted with LM cells.

Table II The effect of CD8+ antibodies on cytotoxic activity of spleen cells from mice injected with a mixture of EO771 cells and LM-IL-2Kb cells.

Mouse immunized with EO771 cells and	EO771 cells			LM cells		
	% specific lysis	% specific lysis + anti-CD8⁺ McAb	Percent inhibition	% specific lysis	% specific lysis + anti-CD8⁺ McAb	Percent inhibition
Media	4 ± 1	3.5 ± 1	12.5	14 ± 3	6 ± 2	54
LM-IL-2 cells	0.7 ± 0.6	n.d.[A]	n.d.	24 ± 0.5	14 ± 3	42
LM-IL-2K^b cells	7 ± 0.6	3.3 ± 2	53	75 ± 6	58 ± 4	23
LM-IL-2K^b/SB-1 cells	12 ± 3	2 ± 1	83	85 ± 3	48 ± 6	44
LM-IL-2K^b/EO771 cells	18 ± 2	0.4 ± 0.2	97	98 ± 1	55 ± 0.6	44

[A]: not determined

Legend: Mice were immunized as described in legend to Table 4. Spleen cells were depleted of CD8⁺ T cells by the addition of 8 ug of anti-Cd8⁺ T cell mAb and incubation on ice for 45 min After washing by centrifugation at 800 x g for 5 minutes, guinea pig complement was added (1:20 dilution in PBS) and incubated at 37°C for 30 minutes. After washing as above, the spleen cells were incubated with Cr-labeled EO771 or LM cells at an effector:target ratio of 100:1. The percent inhibition of the positive response was calculated as described in materials and methods.

CD8$^+$ cells infiltrated the ducts of breast tumors developing in mice injected with a mixture of tumor and vaccine cells

C3H/HeJ mice injected with a mixture of SB-1 cells and LM-IL-2K^b/SB-1 cells survived significantly longer than mice in various control groups (Annual report 2000). C3H/HeJ mice spontaneously develop tumors because they are infected with mouse mammary tumor virus. The resulting tumors were injected into syngeneic mice for tumor passage. The immunity toward SB-1 cells generated by immunizations with LM-IL-2K^b/SB-1 cells was insufficient, however, to fully control tumor growth and the animals died, eventually, from breast cancer.

Immunohistochemical staining was used to characterize the cellular infiltrate in breast tumors developing in mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells. Primary antibodies for mouse CD4 (L3T4), CD8a (Ly-2), CD11b or NK (Ly-49c) cells were used in the analysis. In the experiment, C3H/HeJ mice were injected into the fat pad of the breast with a mixture of 1 X 10^6 SB-1 cells and 2 X 10^6 LM-IL-2K^b/SB-1 cells, and i.p. with 2 X 10^6 LM-IL-2K^b/SB-1 cells alone. The mice received two subsequent i.p. injections and two subsequent injections into the same breast as first injected with equivalent numbers of LM-IL-2K^b/SB-1 cells alone. One week after the last injection, the mice were sacrificed and the immunohistochemical staining reactions were performed on the breast neoplasms. As indicated (Table III), large numbers of cells reactive with CD8 antibodies infiltrated the epithelial ducts of the breast tumors in mice injected with the mixture of SB-1 cells and LM-IL-2K^b/SB-1 cells. Lesser numbers of CD8$^+$T cells

were present intraductally in the tumors of mice injected with SB-1 cells alone (Table III). There were no apparent differences between the numbers of $CD4^+$, $CD11b^+$ or NK cells in breast neoplasms of the treated and untreated groups (Table III).

Table III Immunohistochemical staining of breast neoplasms in mice injected with SB-1 cells and LM-IL-2Kb/SB-1 cells.

	Infiltrating Cells			
	CD4	CD8	CD11b	NK
Injected with SB-1 and LM-IL-2K^b/SB-1 cells	1.1 ± 0.9	9.9 ± 3.4	6.5 ± 3.0	0.4 ± 0.5
Injected with SB-1 cells alone	2.0 ± 1.6	0.9 ± 1.4	8.0 ± 2.0	< 0.1 ± 0.1

Legend: C3H/HeJ mice were injected into the fat pad of the breast with a mixture of 1 X 10^6 SB-1 cells and 2 X 10^6 LM-IL-2K^b/SB-1 cells in a total volume of 200 ul. At the same time the mice received an injection i.p. of 2 X 10^6 LM-IL-2K^b/SB-1 cells in 200 ul alone, followed by two subsequent injections at weekly intervals of 2 X 10^6 LM-IL-2K^b/SB-1 cells i.p. and 2 X 10^6 LM-IL-2K^b/SB-1 cells into the fat pad of the same breast as first injected. As controls, other naive C3H/He mice were injected according to the same protocol with equivalent numbers of SB-1 cells into the breast alone, without subsequent injections. One week after the last injection, histologic sections were prepared for immunohistochemical staining with CD4, CD8, CD11b or NK mAbs, as described in the Materials and Methods section. The data represent an examination of five high powered fields per each of eight slides by three independent observers. The number of positively staining cells in the epithelial ducts was determined.

P < .001 for difference in number of $CD8^+$ cells in tumors of mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells and mice injected with SB-1 cells alone.

P for difference in number of $CD4^+$, CD11b or NK cells in tumors of mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells and mice injected with SB-1 cells alone, not significant.

The same protocol as described previously was followed to characterize the cellular infiltrates in epithelial ducts of tumors forming in C57BL/6J mice injected with a mixture of EO771 cells and LM-IL-2K^b/EO771 cells. Similar $CD8^+$T cell-infiltrates were present in breast tumors developing in C57BL/6J mice injected with a mixture of EO771 cells and LM-IL-2K^b/EO771 cells. Lesser numbers of $CD8^+$T cells were present in the epithelial ducts of tumors developing in mice injected with EO771 cells alone. As for mice injected with SB-1 cells and LM-IL-2K^b/SB-1 cells, there were no apparent difference in the numbers of $CD4^+$, $CD11b^+$ or NK cells in the treated and untreated groups (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

a) Isolated DNA from eight spontaneous breast cancer cells arising in C3H/HeJ mice

b) Modified LM fibroblasts to form IL-2. Confirmed IL-2 secretion

c) Modified LM-IL-2 cells to express H-2K^b determinants

d) Transfected LM-IL-2K^b cells with DNA from different breast neoplasms (SB1, SB5b) arising in individual C3H/HeJ mice

e) Treated mice with SB1 breast cancer with fibroblasts transfected with DNA from SB1 cells. Measured tumor growth in these mice and determined that the treatment resulted in anti-breast cancer immunity.

f) Treated mice with SB1 breast cancer with fibroblasts transfected with DNA from breast cancer arising in a different strain of mice (EO771). The treatment resulted in prolongation of survival of mice with SB1 tumors indicating cross reaction of antigens between the two breast tumors.

g) Determined that CD8$^+$ T cells were required for the anti-tumor response in mice immunized with breast DNA transfected LM-IL-2K^b cells.

REPORTABLE OUTCOMES

In partial fulfillment of the requirements for the PhD degree from the University of Illinois – PhD obtained May 2001.

Manuscript in progress.

CONCLUSIONS:

The data presented here indicate that semi-allogeneic fibroblasts modified to express IL-2 and breast tumor antigens, can activate a potent, specific anti-breast immune response. There is evidence for the development of cytotoxicity mediated by $CD8^+$ T cells. This immune activation results in specific long-term resistance to breast cancer cells.

Like other neoplasms, breast cancer cells form TAAs, several of which have been identified (2,3). However, antigens associated with the proliferating malignant cells are insufficiently immunogenic to generate an effective immune response. Proliferating breast cancer cells fail to elicit anti tumor responses that can control tumor cell-growth.

Here, I tested a unique approach toward the introduction of tumor antigens into a host. I combined two classic findings. The first is that transfection of high molecular weight genomic DNA from one cell-type can alter both the genotype and the phenotypic characteristics of the cells that take-up the exogenous DNA. (4-7).

The second finding, is that the genotype of tumor cells differs from normal, nonmalignant cells of the tumor-bearing host. I hypothesized that undefined, altered genes specifying TAAs would be expressed in a highly immunogenic form by a subpopulation of cells transfected with DNA from the breast cancer cells, and that the number of such cells would be sufficient to generate the anti tumor immune response. A fibroblast cell line was chosen as recipients of the tumor-DNA. The cells, maintained in vitro, were readily transfected, and the cell-number could be expanded as required for the immunizations. Like dendritic cells, fibroblasts can act as efficient antigen-presenting cells (8). Our prior experience indicated that class I cellular anti melanoma immune responses were generated in mice with melanoma immunized with cells transfected with DNA from melanoma cells, and that mice immunized with cells transfected with DNA from non neoplastic (liver) cells failed to lead to anti tumor immune responses (9).

In this study, DNA was isolated from a breast adenocarcinoma, EO771 cells, and was used to transfect LM cells, a mouse fibroblast cell line of C3H/He mouse origin. To increase their non specific immunogenic properties and to ensure rejection, before transfection, the fibroblasts were modified to express allogeneic H-$2K^b$ determinants, and to secrete IL-2. CTL-mediated anti tumor immune responses were generated in C57BL/6J mice immunized with mouse fibroblasts transfected with DNA from EO771 cells. The first appearance of tumor was delayed and the mice survived significantly longer than mice in various control groups, including mice injected with EO771 cells and LM-IL-$2K^b$ cells transfected with DNA from B16 cells, a melanoma cell line of C57BL/6 origin. Immunohistochemical staining of breast neoplasms in the treated animals revealed the presence of numerous $CD8^+$ cells infiltrating the ducts of the tumor. Some of the mice injected with EO771 cells and the modified fibroblasts transfected with DNA from EO771 cells appeared to have rejected the breast cancer cells and survived indefinitely. The requirement for DNA-transfection in generating the cellular immunogen was indicated by the finding that immunizations with non DNA-transfected LM-IL-$2K^b$ fibroblasts failed to generate immunity toward the breast cancer cells. Systemic immunity toward EO771 cells was generated in mice immunized with the DNA-transfected cells. Treated mice were partially resistant to a second injection of EO771 cells.

I interpret these findings as indicative of the expression by the transfected cells of undefined TAAs that characterize the breast cancer neoplasms. Analogous to earlier reports (10), the immunogenic properties of the TAAs were enhanced if they were expressed by IL-2-secreting cells that formed both allogeneic and syngeneic MHC-determinants. The failure of transfected cells that expressed syngeneic MHC-determinants alone to induce an anti breast cancer response reaffirmed the important role of allogeneic determinants in the cells' overall immunogenic properties.

The cellular immunogen was prepared by genetically modifying a fibroblast cell line. Fibroblasts express class I MHC-determinants along with B7, a co stimulatory molecule required for T cell activation (11). Modification of an antigen-presenting cell line, rather than modification of cells from the primary neoplasm, had additional, important advantages. Genetic alteration of the fibroblasts was readily accomplished, and the cells proliferated under standard cell culture conditions. Specialized growth factors were not required. Unlike protocols that require the establishment of a tumor cell line, a technically challenging prerequisite if the cells are to be genetically modified, breast cancer DNA was obtained primary neoplasms taken directly from tumor-bearing mice. It is likely that the quantity of DNA required to generate the vaccine could be obtained from a small quantity of tumor.

Surprisingly, a sufficient proportion of the transfected cell-population appeared to express the products of genes specifying TAAs to induce the anti tumor immune response. My observation that anti tumor immune responses followed immunizations with the transfected cells may be an indication that multiple, and possibly large numbers of immunologically distinct TAAs, the products of multiple genes, were present within the population of breast cancer cells.

The results reported here raise the possibility that a fibroblast cell line that shares identity at one or more MHC class I alleles with the cancer patient may be readily modified to provide immunologic specificity for TAAs expressed by the patient's neoplasm. The data suggest that an optimum response can be obtained if the cellular immunogen is prepared using DNA from the patient's own tumor. Transfection of the cell line with DNA from the neoplastic cells may provide a practical alternative to the modification of autologous malignant cells for the purposes of generating an immunogen useful in the overall management of the patient's disease.

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