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For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease which is not treatable by current approaches. Our goal is to develop vaccination strategies to minimize metastatic disease. We postulate that an improvement in the generation of mammary carcinoma-specific $CD4^+$ T helper lymphocytes will facilitate development of CD8-mediated tumor immunity. To enhance the activation of $CD4^+$ T helper cells, autologous mouse mammary tumor cells are being transfected with syngeneic MHC class II genes plus a variety of costimulatory and adhesion molecules, including B7-1, B7-2, 4-1BB ligand, and ICAM-1. The genetically modified tumor cells should be able to directly present mammary tumor peptides to $CD4^+$ T cells, thereby facilitating their activation. Transfectants are being tested for their tumorigenicity, for their ability to protect tumor-free mice from subsequent exposure to mammary tumor cells, and for their ability to vaccinate against spontaneous metastatic disease in a post-surgical situation.

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(5) INTRODUCTION

For many patients with mammary cancer the primary tumor can be successfully treated by surgical removal, however the long-term prognosis is not favorable because of the high frequency of metastatic disease which is not treatable by current approaches. We are using tumor-specific immunotherapy to curtail the incidence of metastatic breast cancer. Some of the most efficient anti-tumor mediators are tumor-specific $CD8^+$ T lymphocytes. In most cases, for optimal activity $CD8^+$ T cells require "help" from antigen-specific $CD4^+$ T lymphocytes (1). Recent studies indicate that the inability of the tumor-bearing host to reject tumors may be due to a lack of adequate tumor-specific T_h lymphocytes (2-5). We have therefore hypothesized that tumor-specific T_h activity can be significantly improved by generating tumor cells that contain all of the necessary antigen presentation, and accessory and costimulatory molecules such that they are competent for tumor peptide presentation to $CD4^+$ T cells, and thereby facilitate T_h cell activation (reviewed in reference #6). Such genetically engineered tumor cells could be used as vaccines to prevent development of metastatic breast cancer, and thereby enhance a host's tumor-specific immune response.

Because of the initial complexity of the genes being tested, we are using 3 different mouse BALB/c derived mammary tumor models for our studies. The highly malignant 4T07 and 4T1 lines were derived from the spontaneous 410.4 mammary carcinoma and are weakly immunogenic and non-immunogenic, respectively, and the 4T1 subline is spontaneously metastatic while the 4T07 subline is only metastatic if inoculated intravenously (7). Both lines have been adapted for growth in tissue culture (8). The Ts/A-pc mouse mammary carcinoma is also highly malignant and spontaneously metastatic (9).

Our strategy is to genetically modify tumor cells so that they can directly present mammary carcinoma tumor peptides to $CD4^+$ T helper cells, thereby by-passing the requirement for professional antigen presenting cells and making more efficient the presentation of tumor peptides to T helper lymphocytes (reviewed in reference #6). Accordingly, in the *first specific aim* we are using DNA-mediated gene transfer techniques (2) to generate mammary tumor cell transfectants expressing many of the molecules constitutively expressed by professional antigen presenting cells (APC). These molecules include the peptide binding structures or MHC class II molecules, as well as several costimulatory molecules which have been shown to deliver the requisite second signal for T cell activation. The costimulatory molecules to be used include: B7-1 (reviewed in references 10,11), B7-2 (12-15) and 4-1BB ligand (16-19). 4-1BB ligand is a very recently described

costimulatory molecule that is expressed by professional APC such as macrophages and B lymphocytes. Binding of 4-1BB ligand to its counterreceptor 4-1BB on $CD4^+$ and $CD8^+$ T cells transmits a potent costimulatory signal to the T cells resulting in T cell activation. Since 4-1BB ligand appears to function independently or synergistically with other costimulatory molecules (16) it appears to be an excellent candidate for coexpression with B7 genes for enhancing tumor-specific immunity. Mammary tumor cells expressing the cytokines IL-1 (20) and IL-12 (21,22), potent inducers of T_{h2} and T_{h1} lymphocytes, respectively, are also being generated. In addition the adhesion molecule ICAM-1 (23) which is known to facilitate antigen presentation between APC and T cells is being transfected into the mammary tumor lines.

In the *second specific aim* we are determining the tumorigenicity of the transfectants, and their ability to protect the syngeneic host against subsequent challenges of wild type tumor. We will also determine the ability of the transfectants to "rescue" mice carrying established wild type mammary tumors, and identify the helper and effector lymphocytes functional in mammary tumor rejection. In the *third specific aim* we are determining if metastatic mammary cancer can be reduced or prevented by immunization or concomitant treatment with the tumor cell transfectants. This novel tumor-specific immunotherapy approach should significantly improve the host's immune response to autologous breast tumor, and may provide several potential strategies for immune intervention in metastatic mammary cancer.

6. BODY

The first year of the grant period has been spent establishing the mammary tumor system, testing the malignant potential of two of the tumor cell lines, and generating tumor cell transfectants. In addition, studies on other tumor systems on-going in the lab have yielded information that has helped us to fine-tune our proposed experiments.

Transfection of Balb/c derived 4T1 and 4T07 tumor cells with syngeneic MHC class II genes, B7 costimulatory genes, and/or 4-1BBL gene. The first specific aim is to generate mammary carcinoma transfectants expressing syngeneic MHC class II plus costimulatory and/or adhesion molecules. Since the 4T1, 4T07 and Ts/A mammary lines are derived from BALB/c mice, we are transfecting them with syngeneic (ie $H\text{-}2^d$) haplotype MHC class II genes. Mammalian expression vectors carrying the $A\alpha^d$ and $A\beta^d$ MHC class II genes (24), B7-1 (25) and B7-2 (26) genes, and 4-1BBL gene (18) have been obtained, purified, and characterized as previously described for other plasmids (2).

Unfortunately, restriction mapping of the Ab^d gene that was initially obtained from a colleague indicated that the purported Ab^d gene was not the correct gene. As a result we had to obtain a bonifide Ab^d gene from another colleague. Restriction analysis of the secondarily obtained Ab^d gene and the Aa^d gene revealed the appropriate 0.75 kb inserts in the 3.0 kb pCEXV-3 (24). Digests with Bgl1, Xho1, Pst1, or EcoR1 plus Hinc2 also gave the predicted sized bands for the Aa^d and Ab^d plasmids.

Since we propose to generate transfectants expressing multiple genes, it is necessary use several drug selection markers. We have therefore obtained the pSV2neo (encoding G418 resistance; 2), pSV2hph (encoding hygromycin resistance; 27), and pMT3 (dihydrofolate reductase gene [dhfr] encoding methotrexate resistance; 28) plasmids for cotransfection with the MHC class II and other plasmids. Doses of G418, hygromycin and methotrexate for selection of 4T1 and 4T07 transfectants have been optimized as previously described for G418 resistance (2), and are shown in table 1.

Table 1: Optimized drug selection conditions for mammary carcinoma transfectants.

Cell Line	Drug for Selection		
	Hygromycin (μg/ml)	G418 (μg/ml)	dhfr (μM)
4T1	400-800	800	0.5
4T07	800	400-800	0.5

Transfection conditions for the 4T1 and 4T07 mammary tumor cell lines have been optimized by titering the amount of Lipofection (Gibco/BRL) tolerated by the cells during a 24 hour period under the standard transfection conditions (2). Both cell lines titrated at 40 μg Lipofectin/3 ml/10^6 cells/24 hours. Quantities of DNA for transfection were also optimized, and are 2-10μg Aa^d plasmid, 2-10 μg Ab^d plasmid and 1 μg drug selection plasmid (pSV2neo, pSV2hph, or pMT3) for 20-24 hours. We are currently generating the transfectants (4T07/A^d, 4T1/A^d, 4T07/B7-1,4T1/B7-2, 4T07/4-1BBL, 4T1/4-1BBL, etc.) which will be tested by indirect immunofluorescence (2) for expression of the transfected genes.

Monoclonal antibodies for testing class II expression by the transfectants have been obained, purified and tested as previously described for other monoclonal antibodies (2) on known A^d positive cells (MKD6 and 34-5-3 mAbs;

29,30). Monoclonal antibodies for testing B7-1 (1G10 mAb, 25) and B7-2 (31) costimulatory molecule expression have been obtained from Dr. N. Nabavi, U. of S. Carolina. A fusion protein consisting of the 4-1BB protein fused to alkaline phosphatase (16) has been obtained from Dr. Byoung Kwon for testing expression of 4-1BB ligand. With the exception of the 4-1BB fusion protein, all of the mAbs have been extensively tested by indirect immunofluorescence for specificity on MHC class II I-A^d positive and negative control cells. We have also confirmed by indirect immunofluorescence that the 4T1 and 4T07 cell lines do not constitutively express I-A^d class II molecules.

Table 2: Lack of effect of expression of HSA on sarcoma growth.

Immunotherapeutic Cells	Mean Survival Time ± SE (days)
None	29 ± 3.6
Sal/A^k/HSA	30 ± 2.8

The heat stable antigen (HSA) gene therapy experiments of the original proposal have been deleted, because HSA was shown in other tumor systems to not be an effective immunotherapeutic agent. We have decided not to use the heat stable antigen (HSA) gene because results in our lab with a sarcoma tumor have failed to demonstrate an enhancement of tumor-specific immunity following expression of HSA. As shown in Table 2, mice challenged s.c. with the wild type Sal/N sarcoma and then treated with HSA transfected class II positive sarcoma cells (Sal/A^k/HSA tumor) do not show significantly improved survival compared to mice not treated with Sal/A^k/HSA cells. The HSA gene therefore does not appear to significantly improve immunity to established tumor, as compared to the B7-1 costimulatory gene which in a similar experimental protocol mediates rejection of up to 50% of established sarcoma tumors (27). Instead of the HSA gene, we will therefore use the 4-1BB ligand gene (18). As discussed in the *Introduction* of this report, 4-1BBL is the costimulatory molecule that interacts with the recently described counterreceptor 4-1BB. Because of the potent costimulatory activity ascribed to 4-1BBL it appears to be an excellent candidate gene for facilitating the activation of tumor-specific T cells. We have therefore added the 4-1BBL gene to our panel of genes for transfection, and will introduce it into the various mammary carcinoma tumors.

4T07 and 4T1 mammary carinoma lines are being tested for their tumorigenicity, immunogenicity, and metastatic potential. The 4T07 and 4T1

mammary tumor lines have been obtained, and are being titered for their ability to form solid (subcutaneous) tumors, and to metastasize in syngeneic BALB/c mice, using techniques previously described (2, 27). Solid tumor formation is being monitored by s.c. inoculation of graded doses of tumor cells (10^5, 5 X 10^5, and 10^6) into the flank. Developing tumors are measured 2-3 times per week, and mice are euthanized when the tumors reach approximately 2-3 cm in diameter. At this size tumor the mice are not in obvious distress, and hence the tumor incidence studies are being conducted with minimal pain and distress to the test animals. Metastasis formation is being assessed by intravenous inoculation of graded doses of tumor cells (10^4, 5 X 10^4, and 10^5) into the tail vein. Mice are sacrificed 3 weeks post inoculation, the lungs perfused with India ink, and the number of lung metastases counted. The liver and abdominal region of the sacrificed mice are also checked for metastases. In the future we plan to develop an assay for spontaneous metastases by surgically removing primary solid (s.c.) tumors and monitoring the mice post-surgically for spontaneous metastases in the lungs, liver, and draining lymph nodes.

7. CONCLUSIONS

This initial grant year has been spent establishing the mammary carcinoma tumor system, characterizing and generating the necessary plasmids and genes for transfection, and optimizing the transfection conditions for the mammary carcinoma cells. Since most of the reagents are now prepared and the tumors have been characterized, we are in a position to initiate the proposed immunotherapy experiments.

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9. APPENDIX None.