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Associated with Breast Cancer

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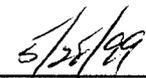
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13. ABSTRACT <i>(Maximum 200 words)</i>  The host immune system can detect tumor-associated antigens (TAAs) existing on cancer cells. While TAA-based immunotherapeutic treatments for the eradication of cancer are being pursued, few TAAs have been defined. We have applied a serologically based strategy called SEREX to the identification of TAAs in the murine colon carcinoma cell line, MC38, and in human breast tumor tissue. We have identified two positive clones in MC38, one as a murine endogenous leukemia proviral envelope ( <i>env</i> ) and LTR sequence, and the other as the murine <i>ATRX</i> sequence. Northern blot analysis demonstrated high levels of expression of <i>env</i> in MC38 cells, whereas expression was absent in normal colonic epithelium. Southern blot analysis revealed several mutations at the DNA level. Northern blot analysis of <i>ATRX</i> demonstrated comparable levels of expression in normal versus MC38 cells, and sequence analysis indicated no mutations. Polynucleotide immunization strategies with these antigens are being pursued. A potential TAA from breast tissue has been isolated and is being characterized. These results demonstrate the potential of SEREX screening to identify tumor antigens which may be utilized as novel targets for cancer immunotherapy.			
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Ray Hampton  
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## **INTRODUCTION**

The identification and characterization of tumor-associated antigens (TAAs) has utility in many areas of breast cancer research. Because humoral immune responses to TAAs exist in mice and humans (1-5), sera can be used as a reagent to detect such antigens. In utilizing an approach termed SEREX for "serological identification of Ags by recombinant expression cloning" (6), we have proposed to use patient sera for the identification and characterization of breast tumor-associated antigens. The importance of understanding TAAs and the role of the immune system in cancer could provide insight into the cellular events leading to tumorigenicity, the immune responses to tumors, and the reasons for failure of such responses during uncontrolled tumor growth. In addition to creating a greater degree of specificity for targeting of tumors therapeutically, identification of TAAs will allow for better detection and diagnosis of cancer in patients. Characterization of TAAs will give scientists a better understanding of tumor biology, including the events which occur in the transformation of a normal cell to a malignant state. In addition, since TAAs are recognized by the host immune system, they can be utilized in deciphering the processes which occur during tumor rejection and tumor. The knowledge gained might someday be of use for the development of immunotherapeutic treatments for cancer.

Our specific aims are as follows:

- 1. Identify TAAs on breast cancer cells by screening with sera from autologous/allogeneic patients.**
- 2. Characterize discovered TAAs.**
- 3. Evaluate the utility of these TAAs in the context of polynucleotide vaccination by assessing immune responses following injection of DNA encoding these TAAs.**

A mouse model system, the MC38 murine syngeneic colon carcinoma cell line, was used to test the SEREX method for the discovery of TAAs expressed on MC38 cells. We know that injection of MC38 cells followed by removal of the tumor cells protects syngeneic mice in a subsequent tumor challenge. Thus TAAs are present on these cells. By identifying and characterizing TAAs in such a mouse model of cancer, we can develop diverse strategies for studying and manipulating the malignant processes that occur in the transformation of normal cells during tumorigenesis. Following the identification of TAAs in this test system, we have begun using sera from breast cancer patients to identify TAAs from breast tumor tissue.

## **BODY**

### **Methods**

#### **Acquisition of tumor and serum samples**

**Mouse model of adenocarcinoma:** The cell lines and immune sera for identification of TAAs in a mouse model were described in our original proposal.

Human samples: We acquired breast tumor samples through the UAB tissue Procurement Facility. We acquired twenty normal human serum samples from samples at UAB's Cancer Center. Serum samples were acquired through the Tissue Procurement Facility as well as from existing samples at the Cancer Center.

### **Construction and screening of cDNA libraries**

cDNA libraries were constructed for the two murine colon carcinoma cell lines, MC38 and MC38-CEA-2, as well as from primary tumor tissue from breast cancer patients. Total RNA was isolated using RNA Stat-60 RNA isolation reagent (Tel-Test B) according to the manufacturer's instructions. Oligo(dT) columns (PolyA-Quik mRNA Isolation Kit, Stratagene) were used to isolate mRNA from total RNA. cDNA libraries were constructed with the ZAP Express Gigapack cloning kit (Stratagene). First strand synthesis was carried out with reverse transcriptase in the presence of a poly(dT) primer with an internal XhoI site as well as a random primer which included a XhoI site at the end. The reaction included 5-methyl dCTP. DNA polymerase I was used to create double-stranded cDNA from the single strands. Following second strand synthesis, EcoRI adapters were added by ligation, and the cDNA was restricted with XhoI. cDNA fragments were cloned into the directional lambda ZapExpress vector, packaged and used to infect *E.coli* cells, through which plaques are formed. As a preliminary characterization of the libraries, inserts from twenty random plaques were amplified by the polymerase chain reaction (PCR) and demonstrated insert sizes ranging from approximately 300 bp to 3.5 kb with greater than 90% recombinants.

In screening for immunogenic peptides expressed from the cDNA libraries, Stratagene's goat anti-mouse secondary Ab + conjugated alkaline phosphatase picoBlue immunoscreening kit was used. This kit is designed to screen phage plaques that are transferred onto nitrocellulose membranes. Recombinant plaques were plated at a density of approximately 20,000 plaques per 150 mm plate, and protein expression was induced using nitrocellulose filters saturated with 10 mM isopropyl b-d-thiogalactoside (IPTG). Filters were removed from the plates, washed three times with Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl) and 0.05% Tween 20 (Sigma), blocked with 1% BSA in TBS and incubated for one hour with sera from patients or immunized mice. Primary sera were preabsorbed with *E.coli* phage lysate (Stratagene) and diluted 1:250 prior to screening. Filters were then washed three times with TBS-Tween 20 (TBST) to remove unbound antibody, followed by incubation for one hour with alkaline phosphatase-conjugated goat anti-human or anti-mouse IgG secondary antibody at a dilution of 1:5,000. Following three washes with TBST and one wash with TBS, immersion of the membranes in the color-producing substrates NBT and BCIP was performed to detect reactive plaques. Positive plaques were purified to clonality for further study.

### **Isotype analysis**

To assess the maturity of the immune response generated to discovered antigens, isotyping kits (Southern Biotech) were used. Positive plaques from the MC38 cDNA libraries were plated as above, filters were incubated with primary mouse serum, washed,

and incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody specific for either IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM. Similarly, positive plaques from the human breast tumor cDNA library were plated, filters were incubated with primary patient serum, washed, and incubated with alkaline phosphatase-conjugated goat anti-human secondary antibody specific for either IgG1, IgG2, IgG3, IgA, or IgM.

### Antigen characterization

#### DNA Sequencing

Following purification of positive plaques, phagemid DNA was rescued by *in vivo* excision from the ZapExpress vector using a helper phage system (Stratagene). In this way, cDNA inserts encoding the TAAs were expressed in plasmid form, with T3 and T7 promoter sequences directly flanking the inserts. Purified DNA was sequenced by dideoxy sequencing using Sequenase 2.0 and 35-S labeled dATP (USB). Approximately 250 basepairs of sequence were determined at both the 5' and 3' ends using T3 and T7 primers, respectively. Sequences were searched against the National Center for Biotechnology (NCBI) databases using the BLASTN.

#### Northern Blot Analysis

Total RNA was isolated from cultured cells using RNA Stat-60 (Tel-Test B) according to the manufacturer's instructions. To isolate total RNA from C57BL/6 mouse colonic epithelium, a section of colon was isolated, opened longitudinally, and the epithelium was recovered by gentle scraping. Epithelial cells were homogenized in RNA Stat-60 and processed according to the manufacturer's instructions. Approximately eight micrograms of total RNA from each sample were electrophoresed on a 1% denaturing agarose gel and transferred to a nylon membrane (Amersham). The blot was prehybridized for 1-2 hours at 65°C in a solution of 6xSSC, 5xDenhardt's, 100 micrograms/ml sheared salmon sperm DNA, 1 mM EDTA and 0.5% SDS. Hybridization with a 32P-radiolabeled probe was performed overnight under these same conditions.

After hybridization, blots were washed successively in 2xSSC with 0.5% SDS, 2xSSC with 0.1% SDS, and 0.1xSSC with 0.1% SDS. The blots were wrapped in plastic wrap, overlaid with x-ray film, and exposed at -70°C for a period of 1 to 4 days prior to development. To demonstrate relative RNA amounts loaded per sample, the blots were stripped with boiling 0.1% SDS and rehybridized with a probe specific for the beta-actin gene (Stratagene).

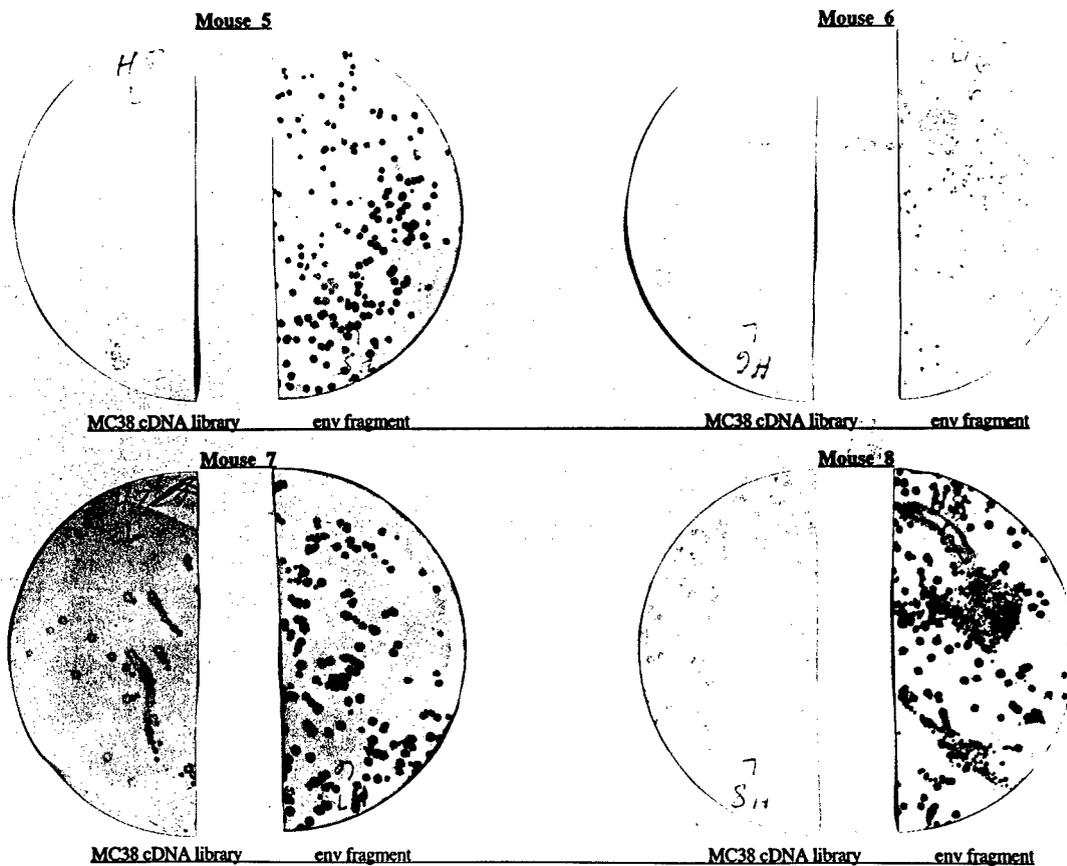
#### Southern Blot Analysis

Genomic was isolated by lysis in 50 mM Tris, pH8, 10 mM EDTA, 10 mM NaCl, 1% SDS and treatment with 100 micrograms/ml Proteinase K overnight at 50°C. Samples were phenol/chlorophorm extracted, and the DNA was precipitated with ethanol and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0. Approximately 10 micrograms of DNA was subjected to digestion with the appropriate restriction enzyme, electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Amhersham). The membrane was hybridized with a labeled probe as above.

## **Results**

### **Identification and characterization of tumor antigens in a mouse model of adenocarcinoma**

We have applied the SEREX approach to the identification of TAAs in the murine colon carcinoma cell line, MC38. Immunization of syngeneic C57BL/6 mice with MC38 cells by three different methods induced protective immune responses with concomitant production of anti-MC38 antibodies. Using these sera to screen an MC38-derived expression library, we have identified a positive clone as a previously described murine endogenous leukemia proviral envelope (*env*) and LTR sequence (see enclosed manuscript) (7). *env* has been defined as a TAA in various murine tumor cell lines. Northern blot analysis demonstrated high levels of expression of this transcript in MC38 cells and several other spontaneously arising and carcinogen-induced tumor cell types, whereas expression was absent in normal colon epithelium. Southern blot analysis revealed several mutations at the DNA level which may be responsible for *env*'s aberrant expression in MC38 cells. Naive C57BL/6 mice injected with DNA encoding *env* mounted strong antibody responses (of subclasses IgG1, IgG2a, and IgG2b) capable of recognizing this protein (Fig 1). These results demonstrate the potential of SEREX screening to identify tumor antigens which may be utilized as novel targets for a polynucleotide immunization approach to cancer immunotherapy.



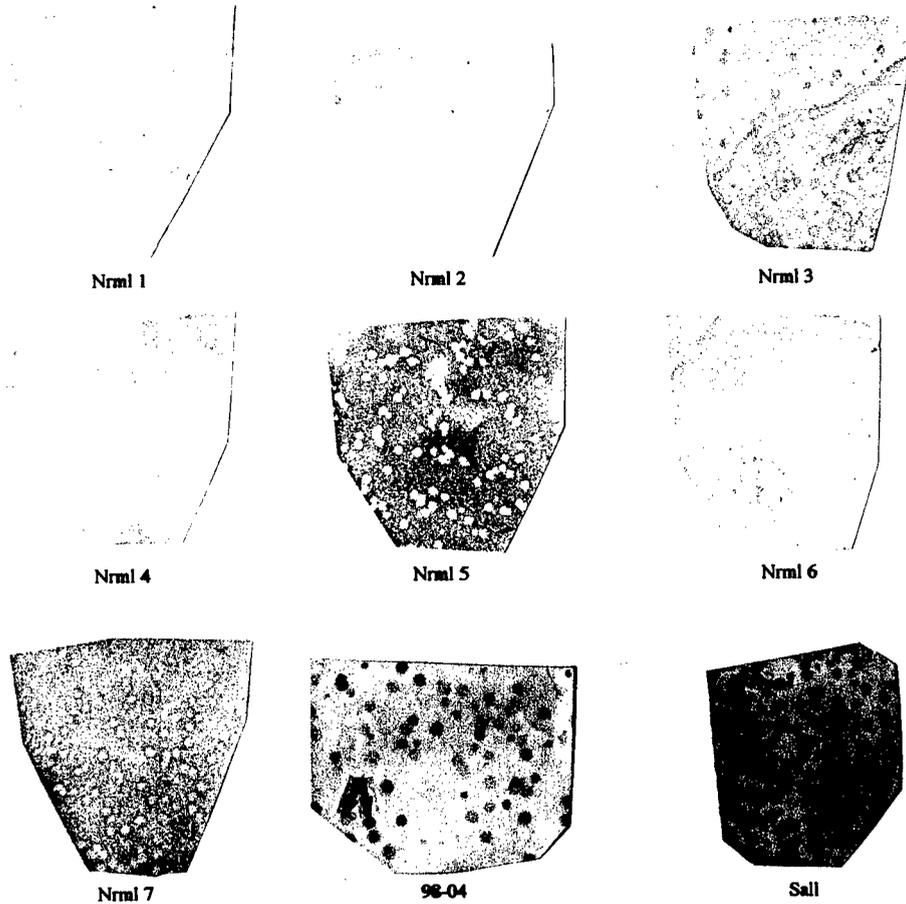
**Figure 1. Assay of humoral immune responses following polynucleotide immunization with a cDNA encoding the 3' region of the murine endogenous retroviral envelope protein.** Ten mice were immunized by DNA injection. Shown are plaques screened with sera from four such animals. Incubation of sera with MC38 cDNA library (left) was used as a negative control for nonspecific binding. Incubation of sera with plaques expressing a portion of the *env* protein (right) resulted in strong reactivity. Normal mouse serum was negative (not shown). Plaques encoding the *env* gene fragment or the library were induced to express protein by IPTG-soaked filters. Filters were lifted, incubated with diluted sera, washed, incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG and visualized by a color reaction. DNA immunization was accomplished by intramuscular injection of 6 x 50 micrograms of plasmid DNA encoding the 1.3 kb fragment of the *env* gene driven by a CMV promoter.

A second tumor antigen recognized by all three groups of immunized mice has been identified in MC38 cells. This 3 kb clone represents a portion of the mouse homologue of the human ATRX gene. ATRX encodes a protein that has potential DNA binding and helicase activities. The mouse and human genes share distinct regions of high homology, and both are thought to be involved in transcription and DNA repair (8). The numerous mutations that have been documented in the human ATRX gene have resulted in a phenotype including mental retardation and alpha-thalassemia (9). The RNA transcript was shown to exist at high levels in B16 melanoma cells and moderate levels in MC38 cells and normal colonic epithelium. Polynucleotide immunization with the 3 kb cDNA resulted in the induction of humoral responses in all immunized animals. Sequence analysis revealed no mutations in the cDNA clone as compared to the normal ATRX sequence.

#### Identification of an antigen from a breast tumor cDNA library

Using autologous and allogeneic sera, we are currently screening cDNA libraries derived from breast tumor tissues (work statement aim 1, task 1). Screening of one such library with autologous serum revealed a positive antigen encoding by a 2 kb cDNA clone. Sequence analysis showed homologies at several regions within the clone with various expressed sequence tags, one of which is from an ovarian cancer cDNA library (accession #AA0745820). Sera from 2 out of 20 patients is reactive with this clone, whereas 10 out of 10 normal serum samples are negative (Fig. 2). The two positive sera demonstrate IgG1 antibody responses to this antigen. Northern blot analysis has demonstrated expression in both normal and tumor tissue (work statement aim 2, task 1).

S3-2



**Figure 2. Assay of humoral immune responses to a potential breast tumor-associated antigen.** Two out of twenty breast cancer patients' sera were found to be positive for this antigen, whereas ten out of ten normal serum samples were negative. Shown are the two positive sera and seven of the negative sera. Plaques encoding the antigenic gene fragment and the library were mixed and plated. Protein expression was induced by incubation with IPTG-soaked filters. Filters were lifted, incubated with diluted sera, washed, incubated with an alkaline phosphatase-conjugated goat anti-human IgG and visualized by a color reaction.

## CONCLUSIONS

The utility of SEREX for identifying and characterizing tumor-associated antigens has been validated. In applying this system to a mouse model of adenocarcinoma, we have identified two distinct tumor antigens. The first, a peptide that is normally not expressed, is a portion of the retroviral env protein. Because this protein has been implicated as a tumor antigen in other cancer systems, this finding is encouraging. The second, ATRX, is a nuclear protein that is expressed in normal tissues. Because the sequence of this antigen is not mutated, the cause of its antigenicity is unclear. We are currently analyzing the location and amount of ATRX protein present in normal and malignant cells. Both murine antigens are being utilized in polynucleotide immunizations to assess the potential for these as tumor rejection antigens. In applying SEREX to the identification and characterization of human breast tumor antigens, we have isolated a positive clone that may represent a shared tumor antigen. Its RNA expression does not seem to be aberrant. As its identity is not yet known, further characterization is underway.

The identification and characterization of tumor antigens is useful for many areas of cancer research and treatment. Studies utilizing these Ags have proven useful in diagnosing cancer and in devising treatments that aid the immune system in guarding against malignancies. Future research in these areas will be important for the development of new immunotherapies for cancer.

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