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PRINCIPAL INVESTIGATOR: Elizabeth A. Repasky, Ph.D.

CONTRACTING ORGANIZATION: Health Research, Incorporated
Buffalo, New York 14263

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Repasky, Elizabeth A., Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Health Research, Incorporated
Buffalo, New York 14263

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

During the three years of our Army support, we have successfully completed the majority of the Specific Aims. We have succeeded in developing a model system for the growth of patients' primary breast tumors in the SCID mouse and this has appeared in print. We have correlated the growth pattern of these tumors in the SCID mouse model with clinical data concerning the diagnosis, treatment and prognosis of the patients. This suggests that ER negative status and/or prior chemotherapy is related to fast growth in SCID mice and poor patient prognosis. Immunohistochemical labelling of tumors grown in SCID mice demonstrates that important tumor markers are expressed in this tumor model (HER-2/neu, CD40 and CD40L, Fas and FasL, CD34, and MHC class I). Tumors have been collected in sequential passages over the duration of our study (months to years) and phenotyping of these tumors is in progress. Preliminary results suggest that with the disease progression in SCID mice, the expression of new antigens may occur while other markers may be down regulated. The effect of whole body hyperthermia (WBH) on reduction of human breast tumor in our model has appeared in print. Our evidence shows that WBH enhances tumor immunogenicity by increasing expression of heat shock proteins and host NK cell killing. Our efforts to identify tumor antigens using human antibodies present in the sera of tumor bearing SCID mice and by the generation of autologous T cell clones show the potential of this model for future development of cancer immunotherapies.

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Elizabeth Ryan

Principal Investigator's Signature

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Date

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## INTRODUCTION

### *Development of the model*

Pre-clinical research of the treatment of human breast cancer has been hampered by the lack of an appropriate experimental animal model in which the growth of primary breast carcinoma biopsies can be studied. Compounding this situation, it is very difficult to obtain enough primary breast tumor cells for most kinds of studies, including antigen identification or RNA and DNA isolation.

Most studies on human breast cancer rely on a few established immortal human breast cancer cell lines. The few cell lines which have been established in long-term culture, and which do grow in immunodeficient mice, most have been obtained from malignant pleural effusions. Although the majority of studies on human breast cancer are conducted on these cell lines, they may bear little resemblance to earlier malignancies found in primary breast carcinoma itself. An animal model that sustains the growth of patient breast tumors could be used not only to study the cellular and molecular properties of various breast carcinoma specimens but also could be used to evaluate the effect of various immuno- or chemo-therapeutic agents or of various factors (such as diet) on breast tumor growth. Moreover, there is a great need for earlier detection markers and better prognostic indicators in the clinical evaluation of human breast carcinoma. Knowledge of biological differences among various primary breast cancers would help to identify such factors and help in the choice of the best treatment modality.

Despite a long-standing effort, human breast carcinomas have proven uniquely resistant to growth in a variety of animal model systems including the anterior chamber of the eye of guinea pigs, lethally irradiated or thymectomized mice and nude and SCID mice (reviewed in the reprint attached to this proposal; Sakakibara et al., 1996) even when the animals are supplemented with estrogen. Apparently, human primary breast carcinoma cells require a very special micro environment for growth that has not previously been replicated in animal models or *in vitro*.

At the time our original Army grant was submitted, we had been testing a variety of protocols attempting to improve the chance of growing human primary breast carcinomas by using SCID mice. Our initial and extensive attempts to engraft human breast tumors in SCID mice by inoculating tumor biopsy tissue or dispersed tumor cells subcutaneously or intraperitoneally were largely unsuccessful, confirming an earlier study by Phillips. However, a successful technique was eventually developed in which surgical specimens of human breast carcinoma were embedded in the gonadal fat pad of SCID mice. Based on our preliminary data (using seven patient' tumors), we submitted the original Army grant.

In this grant, our major goal was to use this model system to develop a model for the study of patients' immunity to their own breast cancer. During the first year of support, we expanded our data base to include well over 40 different patients.

Importantly, we also began to observe the onset of metastasis in the model. For many of our fast growing tumors, we observed metastatic spread to the liver, lung, diaphragm and abdomen. We are currently evaluating mice for bone involvement, since bone metastasis is a very common event in patients with advanced breast cancer.

***Rationale behind the approach***

Cancers of solid tumor origin continue to represent one of the major unsolved problems of modern medicine. For most of the major cancers (which include breast), there has been essentially no improvement in survival since 1930. For most of these cancers, it is possible to control the initial malignancy with surgical excision. Unfortunately, surgical methods are not as successful if the cancer recurs or spreads to other sites. This fact underscores the growing recognition that solid tumor cancer is a systemic disease, requiring systemic treatment. Although there is already a systemic treatment (chemotherapy) applied to most cancer patients as a follow up to surgery, or as neoadjuvant therapy, it is evident that only a small fraction of patients are actually benefiting from this often debilitating treatment.

For at least a century, there has been strong appeal to the notion that an immune response to tumor might exist and develop akin to that observed in infectious diseases. However, implementation of immunotherapies designed to augment the body's own natural defenses in the treatment of human breast carcinoma and other solid tumors has been disappointing. The eventual strategy that is to be developed for cancer immunotherapy will apparently require a far more profound understanding of immune mechanisms and its regulation.

Studies of a variety of animal and human tumor models indicate that the immune response is capable of controlling tumors, particularly in animals that do not have a progressively growing tumor. However, tumors often continue to grow and spread despite the presence of large numbers of lymphocytes (including T, B and NK cells), plasma cells and macrophages in and around the tumor. While there are several possibilities to explain this situation, the escape from immune recognition by human tumors represents one of the most intriguing areas of modern biology. Indeed, in virtually every tumor in which it has been carefully examined, there has been evidence of both immunologic responsiveness and escape from this response in established tumors. Loss of individual MHC class I alleles, loss of cell surface molecules important for susceptibility to apoptotic death induction (Fas), loss of B2 microglobulin expression and peptide transporters required for maturation of the Class I molecule and presentation of antigenic moieties, and production of immunosuppressive cytokines have been identified in human tumors including epithelial neoplasms such as breast carcinoma, cervical carcinoma, and colon carcinoma ( see review in 1). Even more disturbing is very recent data showing that the lymphocytes and other immune elements found within tumors are actually defective in their function.

More than a decade ago, investigators developed methods for the *in vitro* growth of tumor reactive cytotoxic T cell clones (1-4), and the use of these clones in the treatment of tumor bearing animals (5). Indeed, adoptive transfer of *in vitro* propagated cytolytic and helper T-cell clones has been shown to be effective in promoting tumor destruction in a variety of animal models (6) and in human clinical studies (7). The molecular characterization of a variety of T cell growth factors, most notably IL 2 (8) and their use in clinical trials has been of further use in promoting adoptive immunotherapy approaches including the use of lymphokine activated killer (LAK) cells and tumor infiltrating lymphocytes (TIL) in oncology settings (1, 9). While positive results have been seen with such adoptive immunotherapy approaches (most notably in patients with melanoma and in renal cell carcinoma (10)), widespread use of these techniques in common oncology practice has unfortunately been limited due to the large amount of technical sophistication required to grow sufficient numbers of tumor reactive cells ex vivo as well as the necessity of administering toxic amounts of T cell reactive cytokines to patients in order to promote viability and functional reactivity of adoptively transferred tumor reactive T cells. More recently, through the use of gene therapy approaches, investigators have attempted to simplify adoptive immunotherapy protocols by transfecting cytokine cDNA(s) in expressible form (predominantly via retroviral vectors) directly into tumor or lymphoid cells for re infusion into patients (11, 12). However, several additional years of investigation will be required in order to delineate the safety and efficacy of these and related approaches.

One important spin-off of the above mentioned research has been the availability of reasonable numbers of tumor-reactive cytolytic T cells for other aspects of immunological and molecular characterization. Elucidation of the means by which CTL recognize and bind antigen (through the antigen binding cleft of the Class I MHC molecule) has led to the use of tumor-reactive CTL as a means of defining tumor antigens. By stripping peptides from Class I MHC expressed on tumor cells, investigators have identified a number of potential tumor antigens which might hold promise as putative cancer vaccines (13). Indeed, even peptides from subdominant regions of self antigens which are over expressed on tumor cells (e.g. peptides from HER-2/neu) have been shown to stimulate CTL and helper cell responses from cancer patients and in animal models of tumor immunity (14). Despite these advances, the process of identifying tumor antigens which might serve as potential vaccines remains extremely cumbersome given the reliance on large numbers of tumor-reactive CTLs, and sophisticated biochemical approaches for stripping and identifying class I MHC-bound peptides. The translation of this approach is additionally problematic because it requires that tumors be obtained in large quantities that are capable of being made into single cells. This problem is quite significant, particularly in the United States, where surgically excised breast tumors are generally small, and by the time appropriate amounts of the excised tumor have been selected for clinical pathology, available material for preparing cells for *in vitro* culture is usually insufficient and there is little or no chance to have enough material to repeat the experiment.

In our original Army proposal, we proposed to use a newly identified SCID mouse model to help determine effective immunotherapy strategies for patients, since we had found a way to sustain a larger number of patients' breast tumors in SCID mice.

Our earlier experience supported the idea that this SCID mouse model of breast carcinoma could be used to 1) study the patients' own immune response to her tumor and 2) identify strategies to enhance this response in a pre-clinical setting. This is dependent on the inclusion of the patients' own immune cells which have infiltrated the tumor being co-engrafted into the SCID mouse with the tumor specimen.

## OBJECTIVES OF THE ORIGINAL GRANT

1) To characterize the SCID mouse as a model for the growth of human breast cancer and autologous immunocompetent cells and correlate tumor growth parameters with patient's prognosis and expression of predictive markers of tumor malignancy.

2) To demonstrate and monitor specific anti-tumor reactivity of human immunocompetent cells which were co-engrafted with autologous human breast tissue, and correlate these findings to the patient's prognosis and expression of predictive markers of malignancy.

3) To enhance the anti-tumor immunity of human immunocompetent cells in SCID mice by 1) *in vivo* or *in vitro* transfection of human breast tumor cells with genes encoding immunostimulatory proteins and 2) *in vitro* priming of immunocompetent cells with tumor antigens.

## BRIEF SUMMARY OF FIRST YEAR PROGRESS:

As detailed in the October 1995 report, substantial progress was achieved towards the goals of the grant in the first year. Specifically, we had successfully grown over 40 different patients' breast carcinomas in our "GFP model" system. These tumors had grown with remarkably different histologies and growth rates. These differences are probably a reflection of the biological properties of the original tumors. Importantly, we had observed metastatic spread in most of the fast growing tumors within the SCID mice making this model highly relevant to the most critical issues in the study of human breast cancer.

We were in the initial stage of characterizing these tumors for HER-2/neu expression and for DNA content using flow cytometry and will compare these data to the same analysis conducted on the original patients' tumors.

Another important achievement of our first year studies was that we had observed the presence within the tumor-bearing SCID mice of human tumor infiltrating

lymphocytes (TIL) that had apparently co-engrafted into the SCID mice with human breast tumors. We had characterized the lymphocytes for their surface phenotype and had noticed several mice in which there was a great expansion of these TIL. Our results show that a majority of these cells were CD8 positive T cells, but we also observed the presence of plasma cells and CD4 positive cells. These data suggested that this new mouse model for the growth of primary human breast carcinomas may prove a valuable model for study upon both the disease progression and human immunoreactivity against it.

**BRIEF SUMMARY OF SECOND YEAR PROGRESS :**

In the second year of the grant, we made progress in each aim, and toward each goal in the Statement of Work..

Progress toward accomplishing Aim 1: We had collected more data towards the characterization of the growth of human breast carcinomas in our "GFP model" and a publication which describes in detail this model appeared in print (see attached paper titled "Growth and Metastasis of Surgical Specimens of Human Breast Carcinomas in SCID Mice" in The Cancer Journal from Scientific American 1996). We had initiated the evaluation upon correlation of the growth rate of tumors in the SCID mouse with various available clinical data, including nodal status, estrogen receptor (ER) status, and prior exposure to chemotherapy. We had observed that in the group of patients previously exposed to chemotherapy, the tumors seemed to grow fast in comparison to the patients not exposed to chemotherapy. The majority of our fast growing tumors (8 out of 11) were ER negative. However, the growth rate of tumor in SCID mice was not correlated with the presence of axillary nodal metastasis.

Progress toward accomplishing Aim 2: We also began collecting not only peripheral blood from breast cancer patients, but also draining lymph nodes and leukapheresis specimens for the isolation of tumor specific T cells. We had developed several T cell lines and clones which are stimulated by autologous tumor, and can lyse autologous tumor cells. We initiated the study to test the killing efficiency of autologous T cells in SCID mouse model. We had begun to characterize the human antibodies found in the SCID mouse sera using western blots of autologous breast tumor, and other solid tumors in an attempt to identify new antigens.

Progress toward accomplishing Aim 3: Finally, we observed that a long term, low temperature hyperthermia treatment (fever-range exposure) can induce a substantial amount of heat shock proteins in the patients' breast tumor. This treatment also appears to cause a dramatic increase in the killing of tumors by host NK cells. We have been aggressively pursuing this unexpected finding as part of the goals related to the use of heat shock proteins in Aim 3.

## EXPERIMENTAL METHODS

**Animals:** The female CB17-scid/scid mice used for this study were obtained from Taconic Labs or were produced in the breeding colony at Roswell Park Cancer Institute. They were housed in microfilter cages (Lab Products, Maywood NJ). All cages, water and food (Teklad Mills, Wienfield NJ) were supplied after being autoclaved. The cages were maintained in an air conditioned and light controlled (12h/day) room and all handling and operations were done in a laminar flow hood.

**Tumor Implantation:** Fresh specimens of human breast carcinoma were received shortly after surgery. The tumors were sterilely minced (in DMEM F-12 media) to 1.5-2.0 mm-sized pieces with scissors just prior to their implantation into the gonadal fat pad (GFP) of SCID mice. Mice were anesthetized via i.p. injection of 0.4-0.5 ml of Avertin (12.5 mg/ml). A 3 mm horizontal incision was made on the right lower abdomen with scissors and the right portion of the gonadal fat pad was pulled out through the incision. One piece (1.5-2.0 mm) of tumor was placed on the GFP and then enclosed within it by wrapping the GFP around the tumor and suturing it in place (6-0 Dexon, surgical suture). The GFP containing tumor was then replaced, the peritoneum was closed by surgical suture and a surgical stapler closed the skin.

**Tumor growth and Histopathology:** Tumor growth was assessed by palpating the site of implantation. Animals were sacrificed at various times and the tumor was removed for histologic analysis or for passaging of the tumor into other SCID mice. For histopathology, excised tissues were fixed for 24 hours in 10% buffered formalin. The tissues were then dehydrated for 18 hours by an automatic tissue processor and embedded in paraffin blocks. Five micrometer tissue sections were stained with hematoxylin and eosin. All specimens from patients were classified by routine histopathological examination in the Department of Pathology at Roswell Park Cancer Institute.

**ELISA Assay:** The production of human immunoglobulin in the SCID mouse was monitored by a sandwich ELISA assay. Briefly, a series of concentrations of known human Ig or the sera from tumor-bearing SCID mice were incubated with rabbit anti-human IgG (2 g/ml) coated microtiter plates. Peroxidase conjugated affinity-purified rabbit anti-human IgG was used as the secondary antibody to detect human Ig bound to plate. The concentration of human Ig in mouse sera was assessed by measuring 0-phenylenediamine reaction products at OD 490 with an automatic microplate reader (Dynatech Laboratories).

**Immunohistochemical analysis:** Immunohistochemical analysis was performed according to the staining procedure for paraffin sections provided by Vector Laboratories (Burlingame, CA). Briefly, paraffin sections were de-paraffinized and hydrated through xylenes and a graded alcohol series. The tissue sections were then incubated with 0.3% $H_2O_2$ in methanol for 30 minutes to quench endogenous peroxidase activity. The sections were incubated with diluted primary antibodies for 45 minutes and washed 3 times with PBS buffer contained 0.05% Tween 20. The sections then incubated with biotinylated

secondary antibodies for 45 minutes. The Vector ABC kit (Vector Laboratories, Burlingame, CA ) was used for final color development.

**Purification of human IgG from SCID mouse sera:** A 4 ml disposable column was packed with goat anti-human IgG Agarose. The column was then equilibrated with TBS (0.05 M Tris, pH 7.9 with 0.14 M NaCl and 0.02% NaAzide ) until A280 is at base line <0.02). At least 3 ml of the 50% ammonium sulfate cut of the SCID mouse serum (previously dialyzed vs TBS with 10% Sucrose) was applied to the column and the effluent was collected into one tube. The IgG in the sample was incubated for 2 hrs at room temperature to allow the IgG to bind to the gel. The column was then washed with TBS (2 ml/fraction) until A280 is at base line. The unbound fraction was saved for recycling. The bound material was first eluted with 0.2 M Glycine-HCl at pH 2.6 and collected by 1.0 ml fractions into tubes containing 0.2 ml of 0.5 M Tris-HCl at pH 8.8 to neutralize the fraction. A total of 10 ml of Glycine-HCl buffer was used to elute. The column then was eluted with 0.1 M Acetic acid containing 0.5 M NaCl at pH 2.0 and 1 ml fractions were collected into tubes containing 0.18 ml of 1.0 M Tris at pH 8.96. The column was finally washed with TBS containing 25% ethylene glycol to elute hydrophobically bound material. The fractions collected then were checked by SDS-PAGE and Western blot using anti-human IgG as a probe. The human Ig containing fractions were pooled and concentrated with an Amicon concentrator and dialyzed vs TBS. The final product was kept frozen in aliquots at -20 $^{0}$C.

**Preparation of tumor lysate and Western Blot analysis:** Human breast carcinoma cell line (MDA-MB-435S) was obtained from American Type Culture Collection. The tumor cells were cultured in DMEM culture medium to confluence with 10% FBS and 10 ng/ml Insulin (Sigma, ST Louis, MO). The cells were then harvested and incubated with a cell lysis buffer (2% NP-40, 2 mM PMSF, 0.2 mM Leupeptin, and 1 TIU/ml aprotinin in PBS) on ice for one hour and centrifuged at 10,000 rpm for 10 minutes. The supernatants were collected as lysates. Tumors grown in mice were prepared by homogenization of the tumor followed by centrifugation as described above. The samples were mixed with 2X Laemmli sample buffer, boiled for 5 minutes and electrophoresed on 10% polyacrylamide mini-gel of a modified Laemmli formulation. The gel was electrophoretically transferred to polyvinylidenedifluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) using a Mini Trans-blot Cell (BioRad) at 95 V for 1 hour. After transfer, the blot was placed in Tris-buffered saline (TBS) with 10% (W/V) non-fat dry milk and 0.05% Tween-20 (Sigma) for 1 hour to block the non-specific binding of antibodies to the membrane. Test strips were cut from the blot and incubated for 2 hours at room temperature with primary antibodies (sera of the SCID mice bearing human breast carcinoma, 1:1000 dilution) and then incubated with peroxidase conjugated goat ant-human IgG (Zymed, 1:2000 dilution) as secondary antibody. Following each incubation, the test strips were washed 3 times in TBS-0.5% Tween 20. The strips were developed with ECL (enhanced chemiluminescence) reagent (Amersham, Arlington Heights, IL) and then exposed to pre-flashed x-ray film (XAR-5, Kodak).

## RESULT AND DISCUSSION

### Progress toward accomplishing Aim 1:

*A. Correlation of human breast tumor growth in SCID mice with patient clinical status and outcome*

In the last year of this grant, we have completed the study correlating the growth rate of breast tumors in SCID mice with available clinical data on these tumors. The results have been summarized in Table I and II.

The clinical data of these surgical specimens, including nodal status, S-phase fraction, estrogen receptor status, and the patients' outcomes, were collected. The growth rate of tumors in SCID mice was variable and tumors were subsequently classified as fast-growing, slow-growing, or non-engrafting. Fast-growing tumors achieved a size of 1-2 cm within 2-6 months and were passaged at least twice. Tumors were designated as slow-growing if the original explant in the GFP of SCID mice contained growing tumor cells by histopathologic examination but the tumor never achieved a size large enough to be passaged. Explants which did not grow and which did not contain tumor cells upon histological examination were designated as non-engrafting.

Freshly obtained surgical specimens from 76 patients with primary (n=63) or recurrent (n=13) breast cancer were implanted into the gonadal fat pad of SCID mice. 63 patients' primary breast cancers had the median tumor size of 3 cm and 51% were node positive. Overall 74% of tumor were high grade. Fast growing tumors were ER (+) in 43% of patients. Two-thirds of patients in this group were node negative. Forty-three percent of these patients developed recurrence after 12 months of follow up. Conversely, slow and no growth tumors were ER (+) and node (+) in 2/3 of the patients. Forty percent of patients with slow or no growth tumors developed recurrence after 36 months of follow up. Twenty-two percent of patients in the fast growth group were dead within 12 months compared to the slow and no growth groups in which a similar number died within 3 years.

Tumor samples from a subset of the above cases were analyzed by flow cytometry to determine the expression of HER-2/neu and TGF-α on cytokeratin (+) cells which was used as the epithelial cell marker. HER-2/neu and TGF-α were expressed in fresh tumor specimens of most patients who had slow or no growth tumors. The same marker expression was also assessed in the fresh tumor from 6 patients with fast growing and none of the patients expressed these markers (Table II).

The results of our preliminary studies demonstrate that patient derived breast cancers with different growth rates can be identified using the SCID mouse model. The expression of HER-2/neu and TGF-α as determined by flow cytometry could be a useful marker for prediction of disease progression. Furthermore, tumor growth rate in the

SCID mouse may prove important to predict survival and recurrence in short term follow-up.

*B. Expression of tumor markers in original surgical specimens and in mouse bearing tumors by immunohistochemical analysis.*

1) HER-2/neu expression (Figure 2): Our data shows that both surgical specimens and the tumor grown in SCID mice stained HER2/neu positively. However, HER2/neu was strongly expressed in fast growing tumors in comparison to the original surgical specimens. We have also observed a different staining pattern in different individual tumors. For example, in some tumors, HER-2/neu stained the intracellular plasma and in other tumors, the same marker was present on cell surface. More interestely, this staining pattern was maintained in passaged tumors. Further investigation is needed to reveal the significance of this interesting phenomenon. Cytokeratin staining was used to identify the epithelial cell origin of tumor cells (Figure 1B)

2) CD40 and CD40L expression (Figure 1D, 1F): Our preliminary results show positive staining of CD40 and CD40L on both surgical specimens and the human breast tumors grown in SCID mice. However, the staining of CD40 and CD40L in passaged tumors was present at reduced levels in comparison to the surgical specimens. We are currently collecting more data to confirm any existing expression patterns.

3) Fas and FasL (Data not shown). We are in the initial stage of our Fas and FasL study. We have observed the positive staining of Fas and FasL in several tumors. This is in accordance with the reports in the literature. Further study is needed to confirm these results.

4) CD34 expression (Figure 3): To determine whether the vasculature of human tumors grown in SCID mice is derived from mouse endothelial cells, we stained tumors with anti-human CD34 (a marker for human endothelium). Our study showed negative staining on the section from passaged tumors in comparison to strong positive staining in surgical specimens. This indicates that mouse blood vessels support human tumor growth in the GFP of SCID mice.

5) MHC class I molecule expression (Data not shown). Our preliminary study on expression of MHC class I molecules in surgical specimens shows that some tumors stained MHC class I molecule positively and some showed negative staining (data not shown). This staining pattern may prove important regarding prediction of the growth rate of these surgical specimens in SCID mice, since activation of mouse NK cells, which are the important immunoactive cells remained in SCID mice, can be triggered by loss of MHC class I molecules on the surface of tumor cells. We are currently evaluating the expression of this molecule in the passaged tumors in comparison to the original surgical specimens.

The above results suggest that passaged tumors may express new tumor markers that were not present in initial tumor and that some markers may be down regulated in with repeated passages (with the disease progression).

**Progress toward accomplishing Aim 2:**

*A. Evidence of human lymphocytes in tumor -bearing SCID mice:* In the early stages of characterizing the "GFP model", we noticed the presence of lymphocytes and plasma cells in the SCID mice engrafted with human breast tumors (Figure 4). The majority of these lymphocytes are of human origin as demonstrated by immunohistostaining with human CD45 (Figure 1E). Often, these cells can be found distributed around the periphery of tumor cell clusters in animals in which the tumor has failed to thrive.

*B. Human Ig production in the sera of tumor-bearing SCID mice:* We have analyzed the sera of tumor-bearing mice and found the presence of human immunoglobulins in a majority of the mouse sera at a range of 0.5-30 mg/ml. However, we did not see a difference in human Ig production between the groups of fast growing tumors and slow or no-engrafting tumors (Figure 5). Our kinetic studies show that while human IgG can be detected for more than 6 months after tumor implantation, the level of human immunoglobulin in tumor-bearing mice slowly decreased with time (Figure 6). We also noticed that human Ig production decreased with the tumor passages (data not shown).

*C. Purification of human Ig from the sera of tumor-bearing mice:* We have recently isolated human IgG from the sera of tumor-bearing mice, using goat anti-human IgG Agarose (Figure 7). These antibodies will be used to further identify tumor antigens in tumor lysates by Western blot analysis and to detect shed tumor antigens present in the sera of SCID mice bearing passaged tumors by immunoprecipitation analysis.

*D. Identification of tumor associated antigens (Figure 8):* In last year's report, we showed tumor antigens present in tumor grown in SCID mice can be recognized by human IgG in the sera of tumor-bearing SCID mice. Since then, we have performed the Western blot analysis on both tumor lysates of cell line (MDA-MB-435S) and the fractionated serum from tumor bearing mice, using human IgG containing mouse sera as a probe. These Western blots suggest that the breast tumor antigens can be recognized by the human IgG in the sera of tumor -bearing mice. We are currently trying to isolate these potential antigen bands for further identification.

*E. Collection and characterization of patients' T lymphocytes:* During this past year, we continued the isolation and characterization of autologous T lymphocytes collected from either draining lymph nodes or patients' blood obtained by leukapheresis. These cells will be used to do an *in vivo* study to evaluate autologous killing activities and to screen the bacteriophage libraries constructed with tumor cDNA to identify novel tumor antigens.

**Progress toward accomplishing Aim 3:**

In Aim 3, our goal is to develop effective strategies for increasing the immunogenicity of tumors. As described in our last report, we had observed a rapid reduction in tumor volume by exposing the mice to a whole body hyperthermia (WBH) in the fever range ($39^0$C ) for 6-8 hours. Following the initial observation, we did an extensive study to confirm the effect of WBH on the growth of human breast carcinomas in SCID mice and to explore the mechanisms behind it. The paper describing the detail of these studies is in press now and will appear in the May issue of The Journal of Cellular Physiology (see the paper proof and manuscript in appendices)

Using RT-PCR analysis, we have recently observed an increase in the IFN-$\gamma$ production in splenic cells of SCID mice bearing human breast tumors following exposure to $39^0$C for 7 hours (Figure 9). This suggests a potent effect of hyperthermia on mouse NK cells.

**CONCLUSION**

Our work continues to support the notion that the SCID mouse can be an effective model system for the study of various aspects of patients' immune response to breast cancer (including T lymphocyte responses, antibody production and the potential role of NK cells in tumor cell killing following whole body hyperthermia). Key to these studies has been the ability to sustain the growth of primary breast tumor cells (as a source of antigen) and TIL (for antibody production). We believe that the SCID mouse model is uniquely valuable for tumor antigen identification. We expect that this system will prove very important in future tumor vaccine development.

**Publications supported by Army grant**

**Abstracts (see appendix II):**

1. Characterization of autologous immunocompetent cells co-engrafted with human primary breast carcinoma in SCID mice. by Xu Y., Raza-Egilmez S., Sakakibara T., Bankert R.B., and Repasky E.A. Presented at the 1995 AACR Annual Meeting held at Toronto Convention Center, 1995.

2. Effect of long duration, fever-range, whole body hyperthermia on patients' breast carcinomas and the innate immune response. by Burd R., Xu Y., Caligiuri M., Subject J. and Repasky E.A. Presented at 11th Annual Meeting of The Society for Biological Therapy held at Georgetown University Conference Center, Washington, DC, October 23-27, 1996.

3. Engraftment of patients' solid tumors and autologous lymphocytes in SCID mice by Xu Y. and Repasky E.A. Presented at The Keystone Symposia: Cancer immunology and the immunotherapy of cancer. Held at Copper Mountain, CO, Feb. 1-7, 1997.

4. Study of human prostate and breast tumors using a SCID-human chimeric model. Xu Y. Zhao J., Edge S., and Repasky E.A. Presented at Fifth Basic Sciences Symposium of the Transplantation Society, Sept. 6-11, 1997, Chautauqua, NY.

5. Tumor cell apoptosis, NK cell recruitment and tumor vascular changes are induced by low temperature, long duration whole body hyperthermia. by Burd R. Dziedzic T.S., Xu Y., Caligiuri M., Subject J.R., and Repasky E.A. Presented at 12th International Society of Biologic Therapy, Oct. 22-25, 1997, Pasadena, CA.

6. Growth rate and tumor marker expression in human brest cancer. By Hurd T., Repasky E.A., Stewart C., Budnick R., and Edge S. Presented at The Annual San Antonio Breast Symposium, December 1997.

**Reprints (see appendix III):**

Burd R., Dziedzic T.S., Xu Y., Caligiuri M.A., Subject J.R., and Repasky E.A.: Tumor cell apoptosis, lymphocyte recruitment and tumor vascular changes are induced by low temperature, long duration whole body hyperthermia. J. Cell. Physiol. 1998, 176: (will appear in May issue).

Sakakibara T., Xu Y., Bumpers H.L., Chen F-A, Bankert, Arredondo M.A., Edge S.B., and Repasky: Growth and metastasis of surgical specimens of human breast carcinomas in SCID mice. Cancer J. Sci Am 1996, 2:291-300.

**Papers in preparation:**

Xu Y., Zhao J., Edge S, and Repasky E.A.: Characterization of tumor infiltrating lymphocytes (TIL) and human Immunoglobulin in SCID mice implanted with patients' breast tumors.

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# APPENDIX I

# (TABLES AND FIGURES)

## Table I

### Clinical and Biologic Characteristics of Breast Tumors by Growth Pattern in SCID Mice

| Parameter | Fast Growth | Slow Growth | No Growth |
|---|---|---|---|
| # of patients | 23 | 37 | 16 |
| Median F/U (mos) | 12 | 36 | 36 |
| Dead | 22% | 22% | 13% |
| Recurrent Tumor | 43% | 41% | 38% |
| Positive Nodes | 35% | 51% | 44% |
| ER Positive | 43% | 65% | 69% |

## Table II

### Flow Cytometry Analysis of HER-2/neu and TGF

| Parameter | Fast Growth | Slow Growth | No Growth |
|---|---|---|---|
| # of patients | 6 | 17 | 9 |
| HER-2/neu | 0% | 41% | 11% |
| TGFα | 0% | 65% | 78% |
| Node | 67% | 65% | 78% |

Figure 1. **Immunohistochemical analysis of human breast carcinomas (surgical specimens) representative of specimens used for engraftment into SCID mice.** Histochemical analysis was performed on paraffin sections as described in the text. A: Control tissue, unlabelled. B: The identification of epithelial cells in the tumor is confirmed by labelling with anti-Cytokeratin 8 antibodies (DAKO, mAb#35BH11). C and D: Tumor cells are demonstrated to contain the marker CD40 (Santa Cruz, pAb C-20). E: Inclusion of the common leukocyte antigen CD45 in the panel of antibodies identifies the lymphocytes which have infiltrated the patient tumor. Note that the tumor cells are not labelled. F: CD40L, a marker of interest which is also present on lymphocytes, stained both the tumor cells (large cells in the left-lower) and lymphocytes (small cells in the upper right).

Figure 2. **Immunohistochemical analysis of expression of HER-2/neu in the breast tumors grown in SCID mice.** A: HER-2/neu expression was detected by monoclonal antibody (Novocastra mAb # NCL-CB11). Note that although the majority of cells demonstrate positive staining for HER-2/neu, clusters of cells are HER-2/neu negative (arrows). B: Lower magnification of panel A. C: Negative control.

Figure 3. **Immunohistochemical analysis of the vasculature of a tumor grown in a SCID mouse.** A: The vasculature of a surgical specimen is clearly outlined by the endothelial cell marker CD34 (Bectin Dickenson mAb # 347660). B: A breast tumor grown in a SCID mouse does not show similar staining with anti-human CD34 suggesting that the tumor vasculature is derived from mouse endothelial cells. C: A tumor grown in a SCID mouse and stained with H&E indicates that numerous blood vessels are actually present among the tumor cells.

Figure 8. **Identification of tumor antigens by western blot analysis, using the sera of SCID mice bearing breast tumors as a probe.**

A: on the blots 1 and 2, lanes 1 and 3 are the same human breast tumor lysates and lanes 2 and 4 are prostate tumor lysates. The blot 1 was reacted with pooled serum from mice bearing breast tumors, and the blot 2 was reacted with pooled serum of prostate tumor bearing mice. Note that on blot 1, the serum from breast tumor bearing mice recognizes one band on breast lysates only. It also recognizes one band on prostate tumor lysates and one band on both tumor lysates. The blot 2 shows that the serum of prostate tumor bearing mice recognize two bands on prostate tumor lysate and one band on both tumor lysates. These results suggest both shared and specific antigens are present in breast and prostate tumors.

B: Tumor lysate from a breast tumor cell line (MDA-MB-435S) was run on the blot. Lane 1 and 2 were probed with the normal sera from Balb/c mice as negative control. Lane 3 was reacted with pooled serum containing higher level (100 μg/ml) of human IgG and note that two bands were recognized by the serum containing human IgG. Lane 4 was reacted with the serum containing low level (<0.5 μg/ml) of Human IgG.

C: The samples running on the blot were the sera collected from SCID mice bearing fast growing tumors. Lane 1 to 10 were the sera from the mice bearing different human breast tumors and lane 11 and 12 were normal SCID mouse sera. Note that at least 3 bands were recognized in the tumor bearing sera.

Figure 9. **Production of murine Interferon-γ mRNA in response to Low Temperature, Long Duration Whole Body Hyperthermia in spleens of SCID mice bearing human breast tumor #7628:** SCID mice bearing human breast tumor #7628 were treated with WBH for 7 hours. Fourteen hours after the end of the treatment, animals were sacrificed and total RNA was isolated from three spleens from each experimental group using Trizol (GibcoBRL). mRNA was reverse transcribed. PCR was performed using murine specific IFN-γ primers (Clontech ) which amplify a segment of cDNA 365 base pairs in length. The PCR products from non-heated control animals (lanes 2 and 4) and WBH treated animals (lanes 3 and 5) were electrophoresed in a 1.9% agarose gel. Lane 1: 100 base pair ladder; lanes 6 and 7: negative and positive PCR controls, respectively.

Figure 4. **Presence of plasma cells in a lymph-node-like structure derived from a breast tumor explant in a SCID mouse.** A: We often observed lymph-node-like structures formed in the GFP when patients' solid tumors were implanted within the GFP of SCID mice but showed no tumor growth. This is an example of a lymph node like structure formed in the GFP. Some of the nodes consist of pure plasma cells as seen in B and C (at higher magnification).

Figure 5. **Comparison of human breast tumor growth and production of human IgG in SCID mice.** As described in the text, engrafted tumors were classified as either fast or slow growing. These surgical specimens contained infiltrating lymphocytes. Human IgG produced by these lymphocytes was measured in the sera of tumor-bearing mice by ELISA. There was no obvious relationship between the amount of Ig produced and tumor growth.

Figure 6. **Kinetics of human IgG in SCID mice bearing human breast tumors.** 7 surgical specimens of breast tumors were implanted into 28 SCID mice. The mice were bled every two weeks and human IgG in the sera was measured by ELISA assay. Note that Human IgG can be detected more than 4 moths after implantation and the level of human IgG slowly decreases with time.

Figure 7. **Purification of human IgG from the sera of human tumor-bearing SCID mice.** A: Isolation of human IgG. Note the resolution of human IgG purified by affinity chromatography. B: The purified human IgG on Western blot. lane1 represents peak1; lane2, peak 2a+2b; lane3, peak 3a+3b, lane 4 commercial available human-IgG (Pierce) and lane 5 molecular weight standard on 7.5% SDS-gel under non-reducing conditions. The blot was first reacted with rabbit-α human IgG (Pierce, 1:1000), then with goat anti-rabbit IgG-HRP. ( KLP, 1:5000). The arrows show the human IgG bands. 150 kDa band represents intact human IgG, the 56 kDa and the 28 kDa represent the heavy and light chains of human IgG respectively.

Figure 1

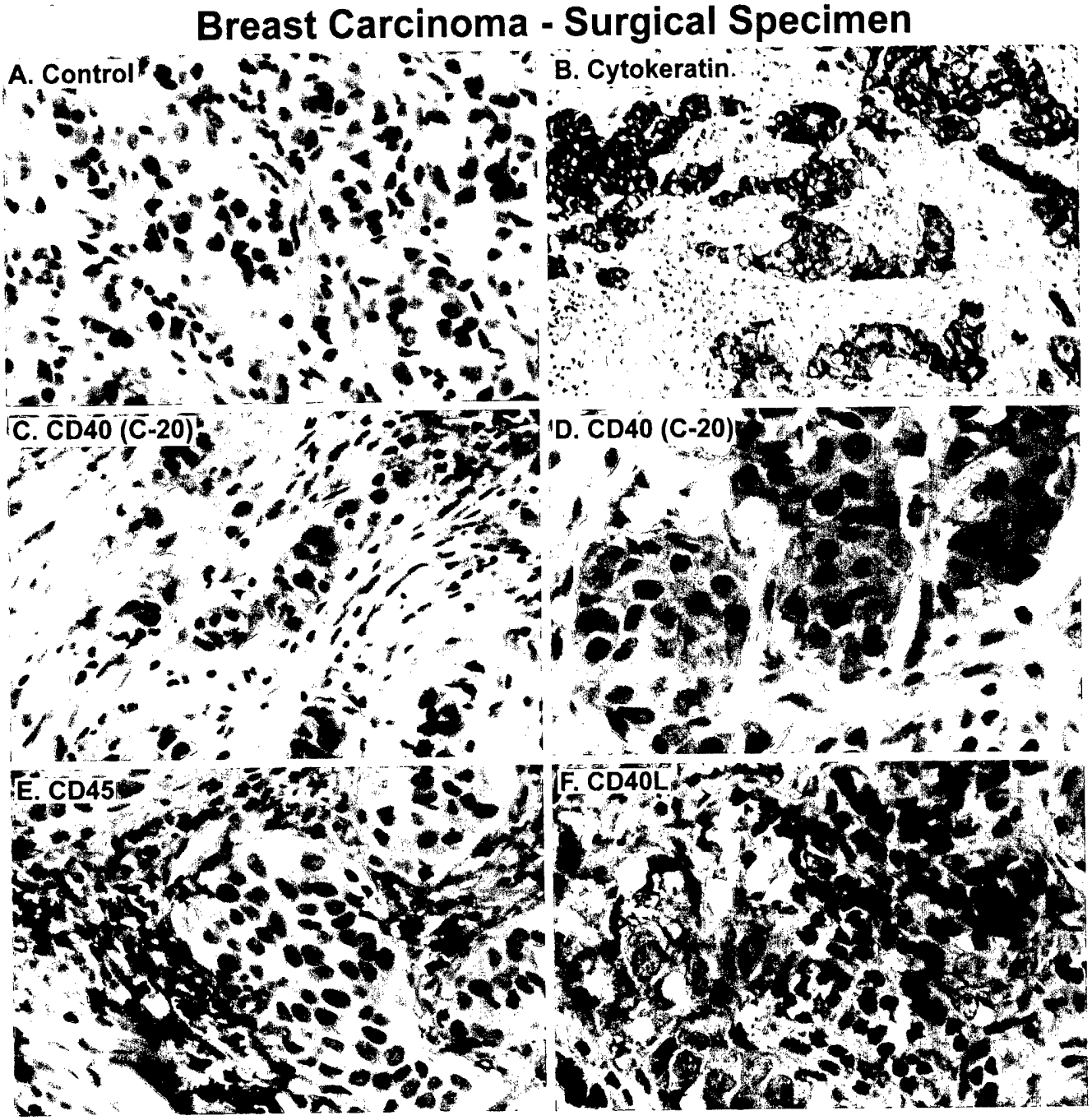
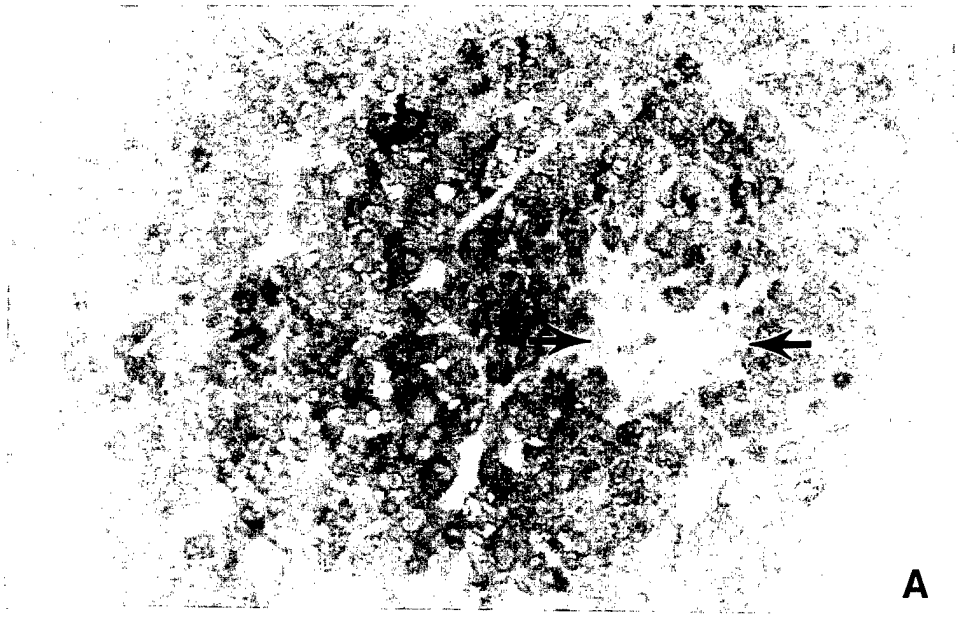
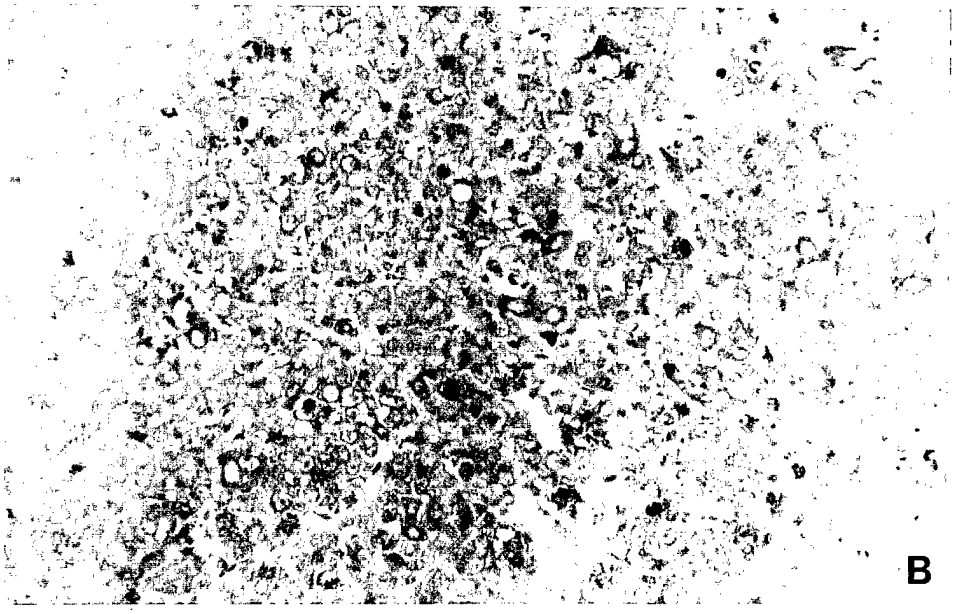

Breast Carcinoma - Surgical Specimen
A. Control
B. Cytokeratin
C. CD40 (C-20)
D. CD40 (C-20)
E. CD45
F. CD40L

Fig. 2



A



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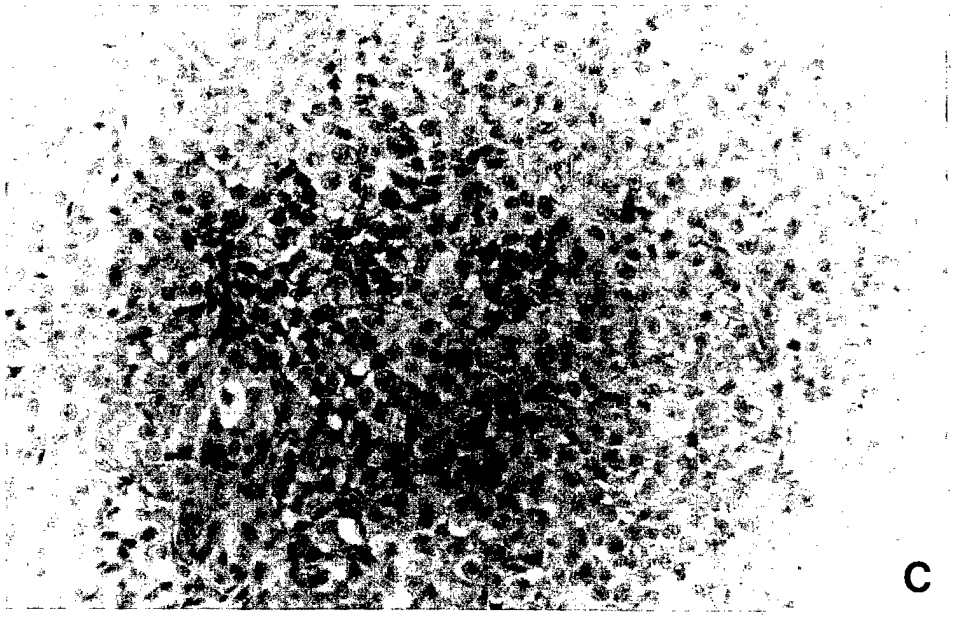


C

Figure 3

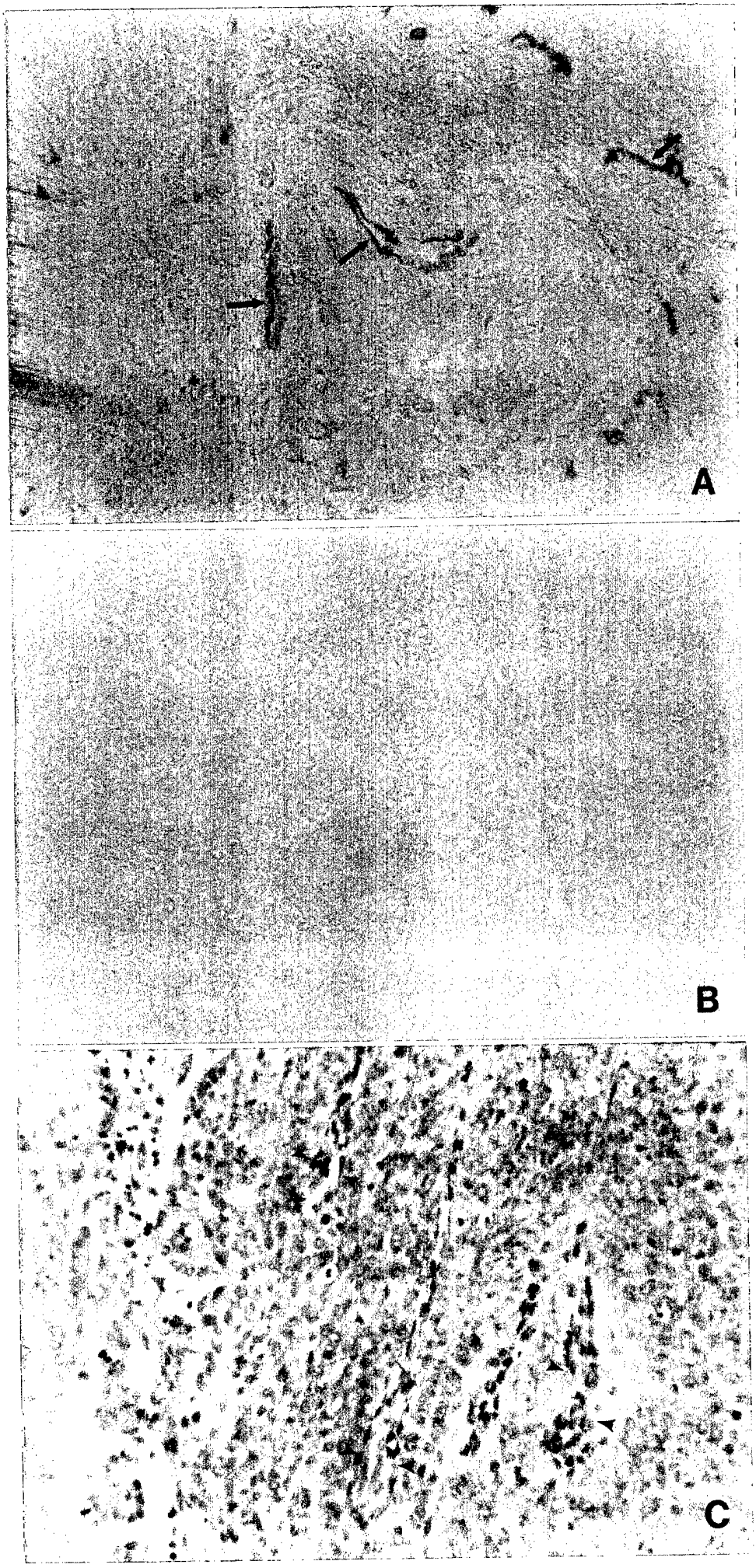


Figure 4

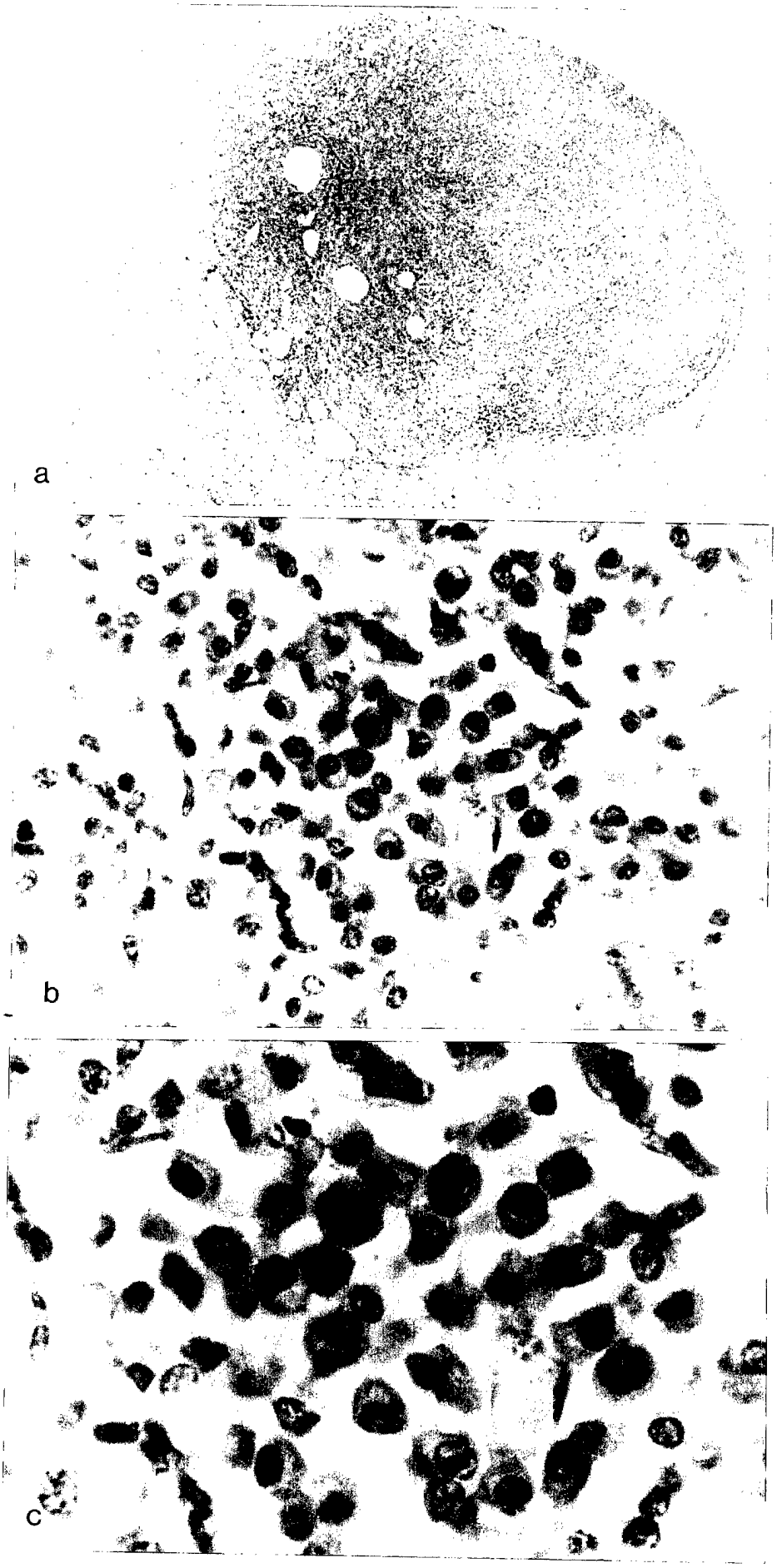


Figure 5

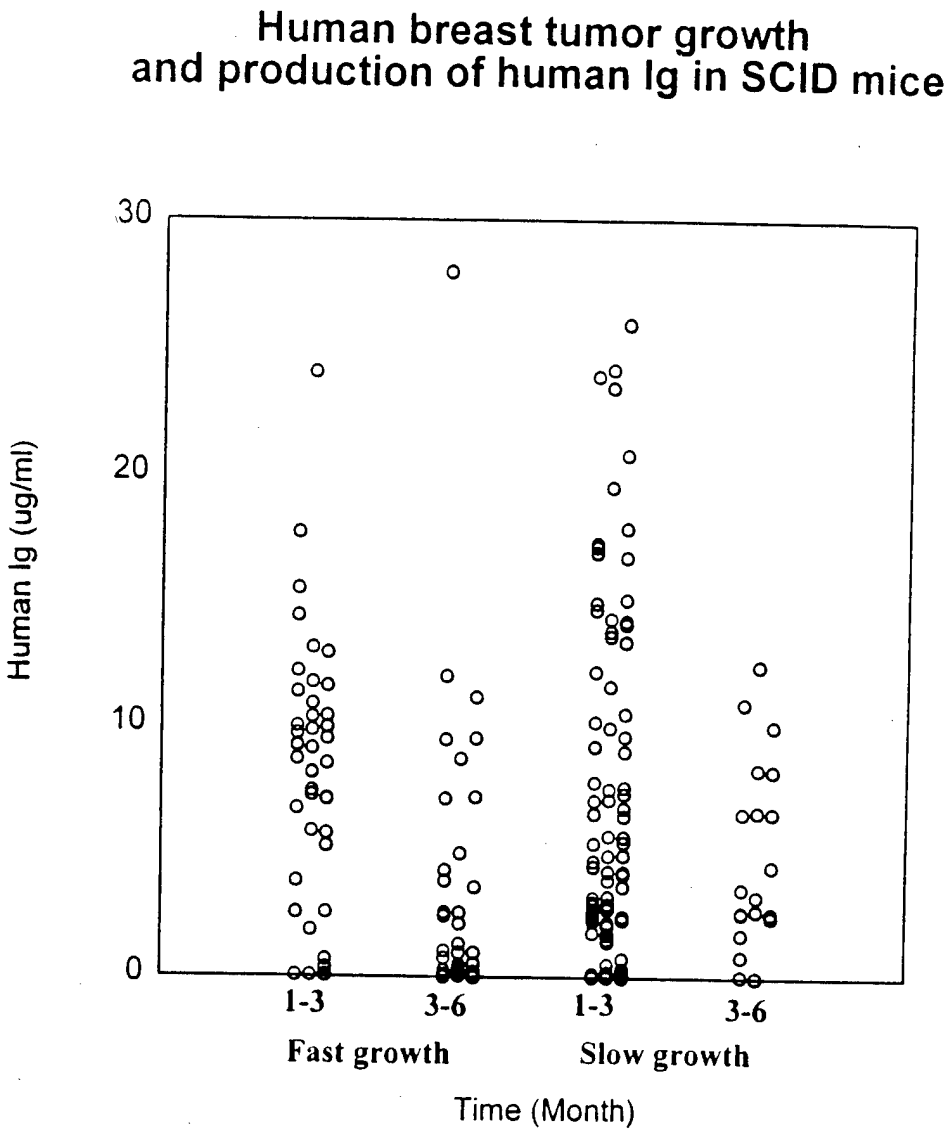

Human breast tumor growth
and production of human Ig in SCID mice
Human Ig (ug/ml)
30
20
10
0
1-3
3-6
1-3
3-6
Fast growth
Slow growth
Time (Month)

**Figure 6**

## Kinetics of Human IgG in SCID Mice Bearing Human Breast Tumors

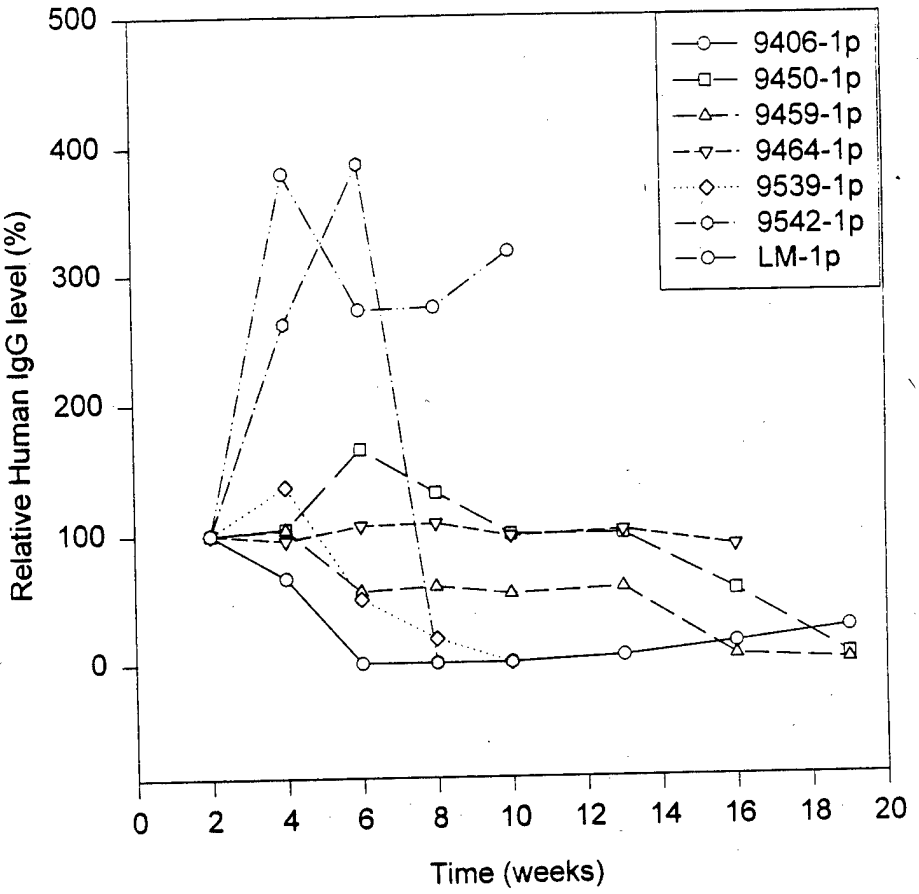


Figure 7A

# Purification of human IgG from SCID mouse sera bearing human breast tumor by affinity chromotography

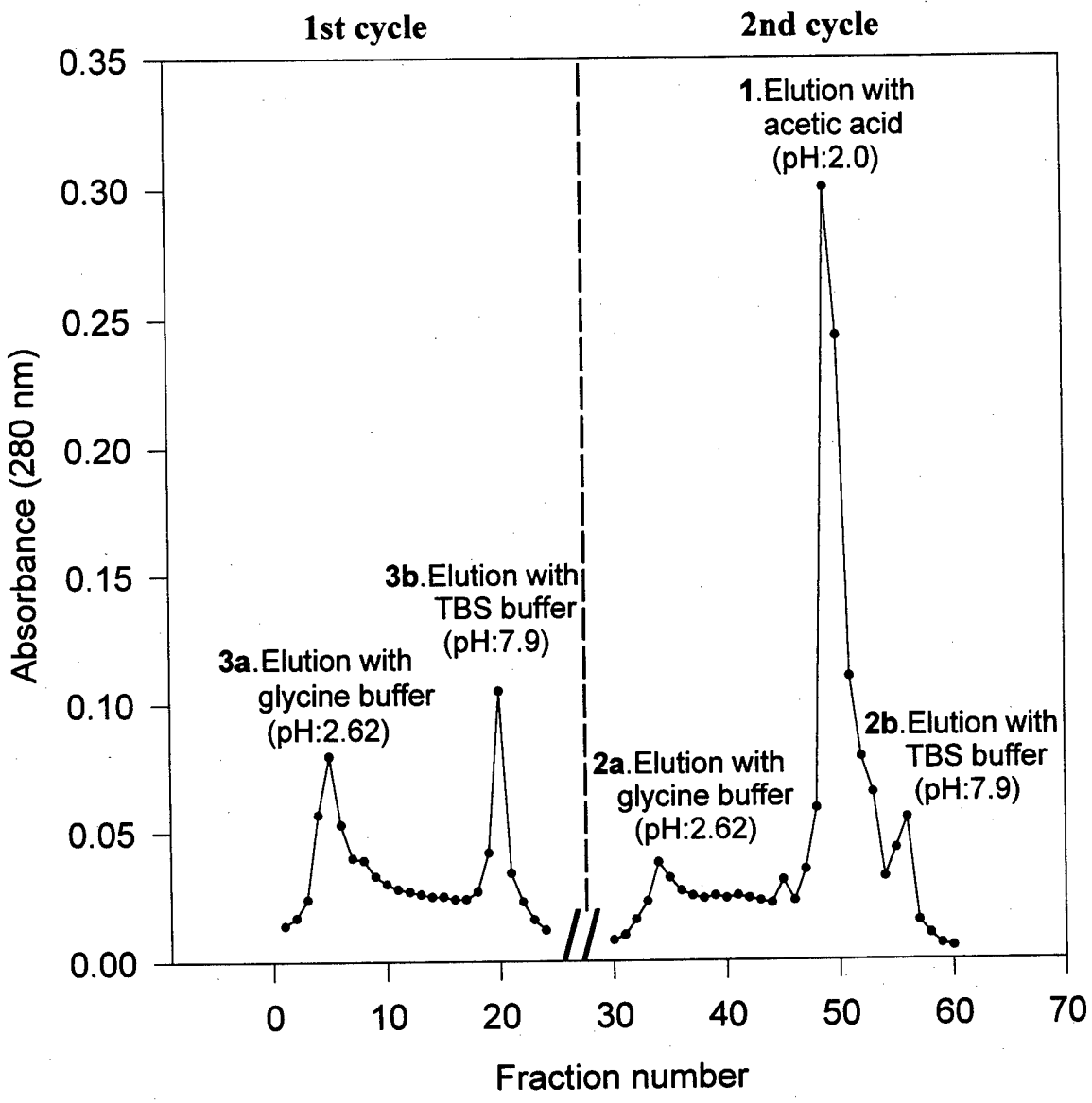


Figure 7B

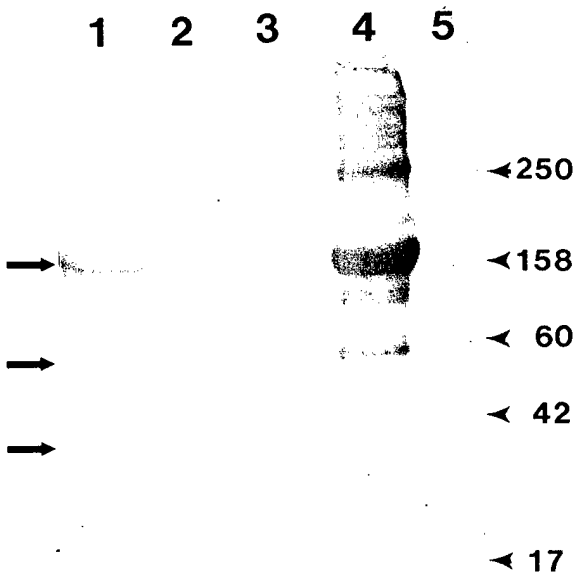
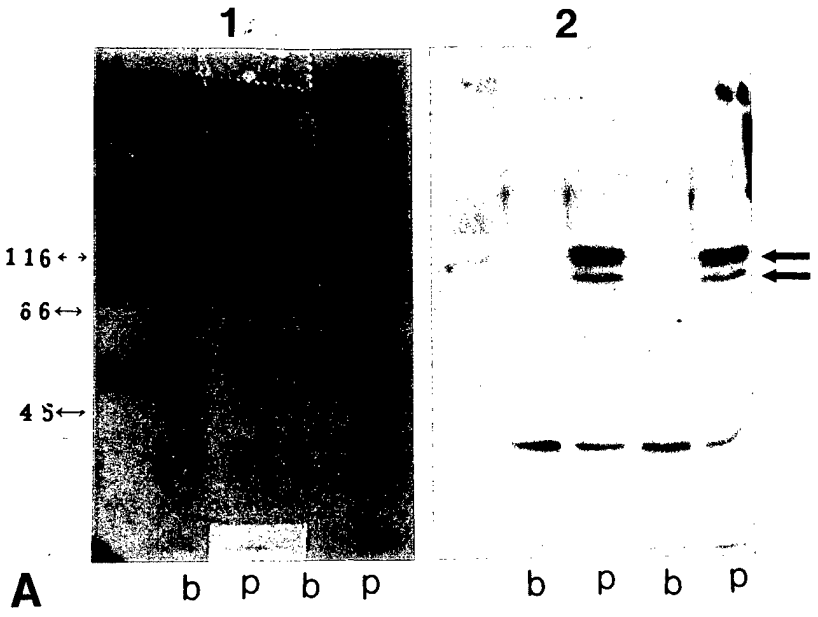


**Figure 8 (A-B)**



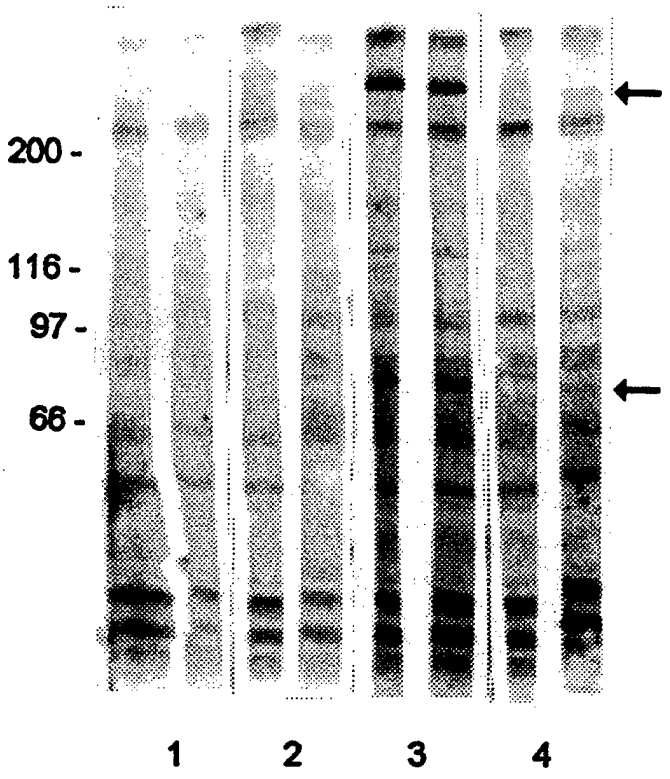
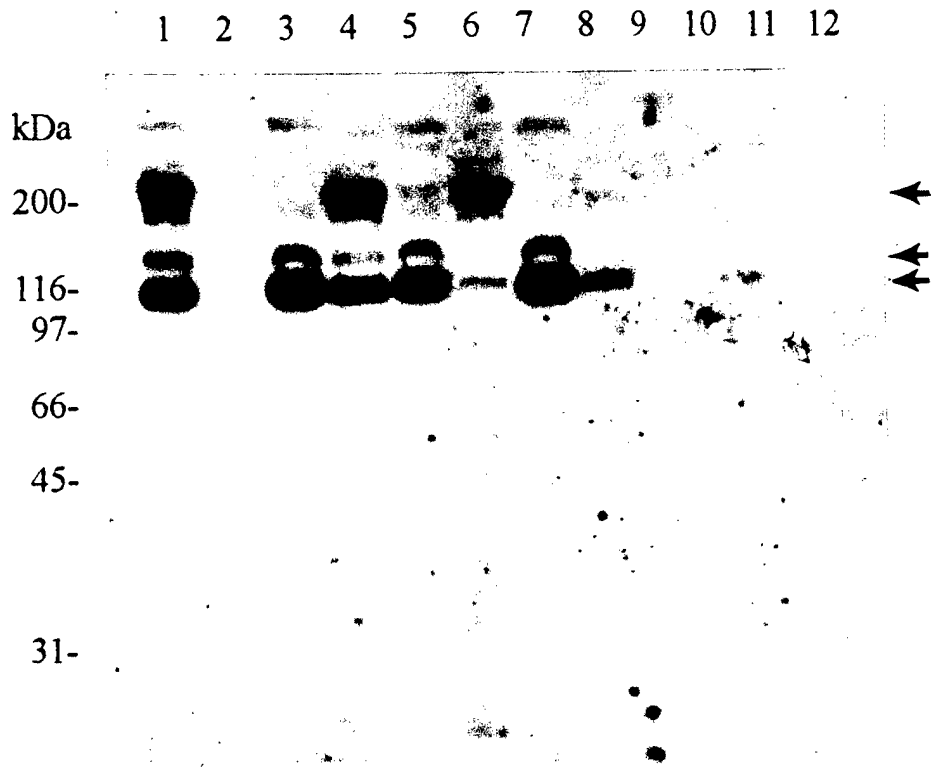
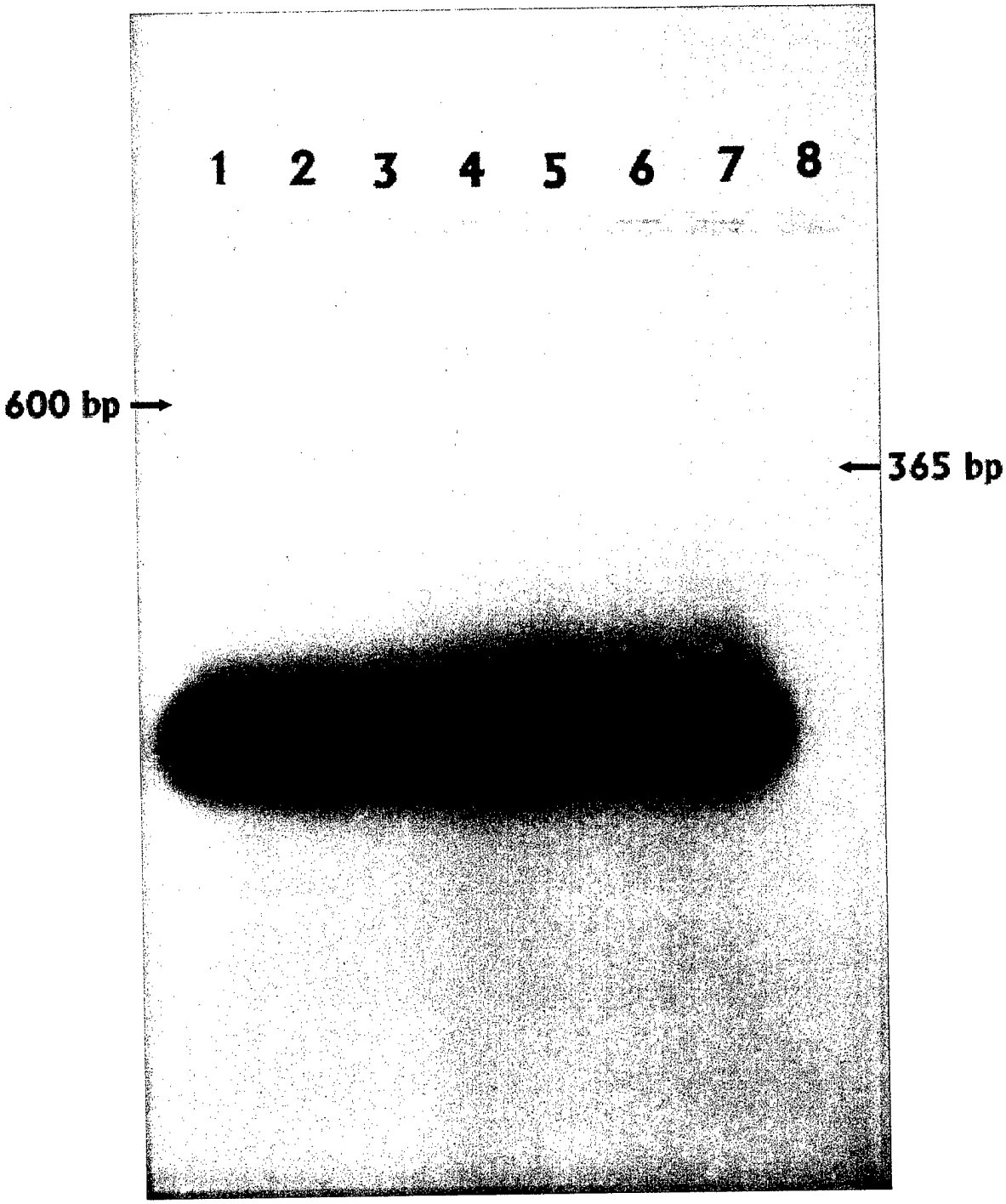



**Figure 8 C**




C

Figure 9



# APPENDIX II

# (ABSTRACTS)

# Coengraftment of Human Primary Breast Carcinoma and Autologous Immunocompetent Cells in Severe Combined Immunodeficient (SCID) Mice

Xu Y., Raza-Egilmez S., Sakakibara T, Bankert R.B., and Repasky E.A.
Dept. of Molecular Immunology, Roswell Park Cancer Institute, Buffalo, NY

We have recently developed a protocol for the growth and passage of human primary breast carcinoma by embedding small pieces of surgical specimen into the gonadal fat pad (GFP) of severe combined immunodeficient (SCID) mice. Using this protocol, we have observed that the histology of the various tumor xenografts and their extent of differentiation can differ considerably, suggesting that breast tumor growth within SCID mice may recapitulate biological properties of the original tumors. We now report the presence of large numbers of lymphocytes and plasma cells that are among the tumor cells; these cells can be visualized for several months following the original engraftment and also in subsequent passages. Immunofluorescent markers indicate that these lymphoid cells are mostly human in origin. ELISA analysis of the sera from engrafted mice shows that the majority of the sera contained human immunoglobulin at range of 1.0 to 300 μg/ml that is probably expressed by the human plasma cells seen within the tumor. Western blotting analysis shows that human breast tumor antigens can be recognized by these antibodies. We believe that this new SCID-human chimera model will prove valuable in the exploration of the pathogenesis of human breast cancer, in the understanding of immunological responses to the malignant cells, and in the testing of new therapeutic approaches for treatment of this disease. **Present at 86 annual meeting of American Association for Cancer Research. March 18-22, 1995, Toronto, Canada**

Abstract submitted to the Eleventh Annual Meeting of The Society for Biological Therapy to be held in the Georgetown University Conference Center, Washington, DC, October 23-27, 1996

EFFECT OF LONG DURATION, FEVER-RANGE, WHOLE BODY HYPERTHERMIA ON PATIENTS' CARCINOMAS AND THE INNATE IMMUNE RESPONSE. R Burd, Y.Xu M. Caligiuri, J. Subjeck and E. Repasky. (Roswell Park Cancer Institute, Buffalo, New York)

We have examined the effects of long duration, fever-range, whole body hyperthermia (WBH) treatment on patients' breast, colon and prostate carcinomas grown in SCID mice. Mice bearing breast tumors implanted into an abdominal fat pad were treated for 8h at 39.5-40$^0$ C (rectal temperature) in a gravity convection oven. Breast tumors treated with WBH had a 40% ($p<.05$) reduction in tumor mass 8d after treatment. We also examined patients' breast, prostate and colon carcinomas implanted subcutaneously. Treatment of each of these tumor bearing mice with WBH resulted in a rapid and substantial reduction in tumor volume coinciding with a significant increase in apoptosis of tumor cells. However, when anti-asialo GM-1 antibody (NK cell inhibitor) was injected prior to treatment, the tumor growth delay was abolished as well as the apoptosis. Human NK cells labeled with a Zynaxis fluorescent dye were injected into tumor bearing mice. Following WBH we observed a large increase in the number of NK cells within the tumors from treated mice, while few NK cells were observed in tumors from non-treated mice. Thus, fever-like WBH may be clinically useful, especially as an adjunct to immunotherapy by its ability to stimulate NK cell activity.

# Engraftment of Patients' Solid Tumors and Autologous Lymphocytes in Severe Combined Immunodeficient Mice

Xu Y. and Repasky E.A.
Dept. of Molecular Immunology,
Roswell Park Cancer Institute, Buffalo, NY

To help develop improved models for the study of solid tumor immunity, we have analyzed the histology of over 250 human breast, prostate, lung and esophagus carcinomas implanted into SCID mice. We have observed that the growth potential and histology of thetumor xenografts and their extent of differentiation and vascularization can differ considerably, suggesting that solid tumor growth within SCID mice may recapitulate biological properties of the original tumors. We have also observed the presence of lymphoid cells among the tumor cells, and at various locations distant from the implantation site. Analysis indicates that these cells are mostly human T and B lymphocytes and plasma cells, and some murine cells. ELISA assay of the sera from engrafted mice shows that the majority of mice contain human immunoglobulin ranging from 5 to 400 μg/ml. Western blotting analysis shows that the human antibodies recognize human breast, prostate and lung tumor antigens. We have also studied the co-engraftment of tumors and autologous lymphocytes isolated from draining lymph nodes and peripheral blood of patients; our data indicate that tumor killing activity can occur in these mice following specific cytokine treatment and whole body hyperthermia. We believe that the SCID-human chimeric model will prove valuable for achieving a better understanding of immunological responses to malignant cells, and in the identification of tumor antigens. (Supported by US Army 17-94-J-4418) **(Presented at Keystone Symposia: Cancer Immunology and the Immunotherapy of Cancer, Feb. 1-7, 1997, Copper Mountain, Colorado)**

# Study of Human Prostate and Breast Tumors Using A SCID-human Chimeric Model

**Yan Xu, Ph.D[1].; Jing Zhao[1], Stephen Edge, M.D[2]. and Elizabeth, A.Repasky , Ph.D.[1]**

Depts. of Molecular Immunology[1], Breast Surgery[2], Urologic Oncology[3]
Roswell Park Cancer Institute, Buffalo, NY, 14263

To help develop improved animal models for the experimental study of human solid tumors, we have studied the growth of surgical specimens of human solid tumors, especially breast and prostate carcinomas in severe combined immunodeficient (SCID) mice. We studied the growth potential and histology of the various patients' tumor xenografts and the extent of differentiation and vascularization. Our study suggests that these biological properties can differ considerably and that the histology of the xenographs is often similar to the original tumors for long periods of time. In addition, we have established several metastatic tumor models from patient's primary breast and prostate carcinomas, in which there is multi-organ metastatic spread. We have also observed the presence of human lymphocytes and plasma cells among the tumor cells. The majority of tumor-bearing mice express human immunoglobulin and these antibodies recognize antigens on Western blots of tumor lysates. We found that sera from human lung and prostate tumor bearing mice each recognize a single band on a variety of solid tumors, including breast, prostate, colon, esophageal, ovarian, and lung, suggesting that common antigens may exist among variety of human solid tumors. Our data also showed that the sera from mice with a given type of solid tumor also recognize one or on more specific bands on that tumor only or on the tumor type only, suggesting the presence of tumor specific antibodies. We believe that this SCID-human chimeric model will prove valuable in studying disease progression of human solid tumors, in searching for tumor antigens and in testing new immunotherapeutic approaches.
{Supported by the Department of Defense (#8570607)} **(Presented at Fifth Basic Sciences Symposium of the Transplantation Society, Sept. 6-11, 1997, Chautauqua, New York)**

Presented at the 12th International Society of Biologic Therapy, October 22-25 1997, at Pasedena, CA.

# TUMOR CELL APOPTOSIS, NK CELL RECRUITMENT AND TUMOR VASCULAR CHANGES ARE INDUCED BY LOW TEMPERATURE, LONG DURATION WHOLE BODY HYPERTHERMIA.

R. Burd, T. S. Dziedzic, Y. Xu, M. A. Caligiuri, J. R. Subjeck, and E. A. Repasky* (Roswell Park Cancer Institute, Buffalo, NY 14263 U.S.A.).

A single treatment of low temperature, long duration whole body hyperthermia (WBH) of mice bearing either human breast tumors or transplantable, syngeneic murine tumors can cause a reduction of tumor volume and/or a growth delay. This inhibition is correlated with the appearance of large numbers of apoptotic tumor cells. Addition of IFN-γ was found to significantly enhance this effect. Our data support the hypothesis that this mild WBH protocol stimulates some component(s) of the immune response, which results in increased anti-tumor activity. Increased numbers of lymphocyte-like cells, granulocytes and macrophages are observed in the tumor vasculature and in the tumor stroma immediately following the WBH exposure. Using a SCID mouse/human tumor xenograft system, we found that both host NK cells, and injected human NK cells were increased at the site of tumor following WBH. Using anti-asialoGM1 treatment in this system, we observed that the tumor cell apoptosis appears to be due almost entirely to the activity of NK cells. Another interrelated hypothesis is that immunoeffector cells have greater access to the interior of the tumor following WBH since we have observed that this treatment causes an obvious expansion in the diameter of tumor blood vessels, which persists as long as two weeks following treatment. Further study of the mechanisms by which mild hyperthermia exerts anti-tumor activity could result in this treatment protocol being used as an effective, non-toxic adjuvant to immunotherapy and/or other cancer therapies.

DO NOT TYPE ON OR PAST THE BLUE LINES

Repasky Elizabeth A

Department of Molecular Immunology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263 USA

(716)845-3133 (716)845-8906 repasky@SC3101.med.buffalo.edu

Presented at The Annual San Antonio Breast Symposium
December 1997

**GROWTH RATE AND TUMOR MARKER EXPRESSION IN HUMAN BREAST CANCER.** Hurd T, Repasky E, Stewart C, Budnick R, Edge S. Roswell Park Cancer Institute and State University of New York at Buffalo. Buffalo, NY 14263.

Introduction: The relationship between clinical outcome in patients with breast cancer and the growth rate of their tumors in the SCID mouse has not been characterized. We hypothesized that the growth rate of patient derived breast tumors in SCID mice correlates with tumor marker expression, recurrence, and survival. Methods: Fresh tumor was obtained from 76 patients with breast cancer (63 primary, 13 recurrent). Nodal status, S-phase fraction and estrogen receptor were determined. Tumor samples from a subset of cases were analyzed by multiparameter flow cytometry to determine c-erb-B2 and TGFα expression in cytokeratin positive cells. Tumor growth rate in SCID mice was measured after implantation in the gonadal fat pad and classified as fast, slow, or no growth. Results: The median tumor size of 63 primary breast cancers was 3 cm. and 51% were node positive. Overall, 74% of tumors were high grade.

**Clinical and biologic characteristics by growth pattern in SCID mice**

| Parameter | Fast growth | Slow growth | No growth |
|---|---|---|---|
| # of patients | 23 | 37 | 16 |
| Dead | 22% | 22% | 13% |
| Recurrent tumor | 43% | 41% | 38% |
| High S-phase | 65% | 59% | 50% |
| Node positive | 35% | 51% | 44% |
| ER positive | 43% | 65% | 69% |

**Subgroup analysis of TGFα and c-erb-B2 expression**

| | Fast growth | Slow growth | No growth |
|---|---|---|---|
| # of patients | 6 | 17 | 9 |
| c-erb-B2 (+) | 0% | 41% | 11% |
| TGFα (+) | 0% | 65% | 78% |
| Node (+) | 67% | 65% | 44% |

Conclusions: 1. The SCID mouse model identified breast cancers with different growth rates. 2. Tumor growth rate in the SCID mouse is predictive of survival but not recurrence in short term followup. 3. TGF-α and c-erb-B2 expression may not correlate with rapid growth rate or nodal involvement.

# APPENDIX III

# (REPRINTS)

# Growth and Metastasis of Surgical Specimens of Human Breast Carcinomas in SCID Mice

Takashi Sakakibara, MD,* Yan Xu, PhD,* Harvey L. Bumpers, MD,[b] Fang-An Chen, PhD, Richard B. Bankert, VMD, PhD, Mark A. Arredondo, MD,[a] Stephen B. Edge, MD,[a] Elizabeth A. Repasky, PhD, *Buffalo, New York*

■ **PURPOSE**

We have studied the growth and metastatic potential of surgical specimens of breast carcinomas engrafted into the large abdominal (gonadal) fat pad of severe combined immunodeficient (SCID) mice. We present results of this study, details of the implantation protocol and histologic characterization of several of the tumor xenografts.

■ **MATERIALS AND METHODS**

We evaluated the growth within SCID mice of 48 breast carcinoma specimens derived from 46 patients (45 primary breast cancers or local recurrences and 3 regional metastatic lymph nodes) obtained from resected tissues at this Institute over a 3-year period. The growth of each transplant was assessed by histologic examination of the xenografts at various times after implantation or upon passage into additional mice.

■ **RESULTS**

We observed that placement of human breast tumors within the gonadal fat pad could result in tumors that grew either rapidly, slowly, or not at all. Of 48 tumors studied, 12 (25%), including one of the three lymph node–derived tumors, grew rapidly enough within some or all of the implanted mice (i.e., the tumors reached a diameter of 2–3 cm within 2–6 months) to allow repeated passage. Metastatic spread to the SCID mouse lung, liver, and/or diaphragm and other sites was observed with the xenografts derived from 8 of these 12 rapidly growing tumors. Tumors in a second category often took from 6 months to over 1 year to only double or triple in size. This slow-growth group consisted of 25 patients' tumors (53%), including the remaining two metastatic lymph node–derived tumors. These xenografts would usually maintain a slow growth rate even upon later passage into new animals. A third category consisted of 11 patients' tumors (23%) that failed to grow at all (i.e., no evidence of tumor growth in any of the mice implanted), as discerned by histologic evaluation at various times after implantation.

Histologic examination of tumor xenografts and metastatic tumors revealed considerable variation in histopathology among the different patients' tumors.

■ **DISCUSSION**

Further examination of the heterogeneous properties of primary human breast carcinomas within SCID mice may provide a simple yet valuable new approach for the long-term study of human breast cancer biology. Importantly, use of the protocol described here can often permit the isolation of substantial quantities of human breast cancer cells for biochemical and molecular analyses. The ability to passage patients' breast tumors into large numbers of mice will permit the preclinical testing of new therapies for the treatment and prevention of this disease. *(Cancer J Sci Am 1996;2:291-300)*

**Key words:** Human breast carcinoma, SCID mice, metastatic breast cancer, gonadal fat pad, xenograft, estrogen.

Most recent experimental studies on human breast cancer rely on only a few established immortal human breast cancer cell lines; in particular, the endocrine-responsive MCF-7 line or the endocrine-independent MDA-MB-231 line. This dependence on only a few cell lines is due to the low success rate for establishing human breast carcinomas either as stable cell lines in vitro or directly as xenografts in immunodeficient mice.[1-5] Among the cell lines that have been established in long-term culture, and that grow in immunodeficient mice, most have been derived from the highly malignant cells found in pleural effusions. Because these cell lines were isolated from only a remarkably small subset of even the metastatic specimens, it is likely that they represent only the most aggressive subpopulation of breast cancer cells and bear little physio-

From the Departments of Molecular Immunology and [a]Surgical Oncology, Roswell Park Cancer Institute, and [b]The Surgical Group Practice of the Faculty at the State University of New York at Buffalo, Buffalo, New York.

* Each author contributed equally to this research.

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Reprint requests: Elizabeth A. Repasky, PhD, Department of Molecular Immunology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263.

logic resemblance to those found in primary or locally recurrent breast tumors.[1-3] Therefore, although the majority of cellular and molecular studies on human breast cancer are conducted on these long-term cultured, stable cell lines, the data derived are limited to the very latest stages of disease progression. Because many of these cells have been in continuous culture for many years, it is also likely that these cells may have undergone significant phenotypic changes in response to selection pressures of in vitro culture, rendering them even further distinct from patients' breast tumor cells.[2] The potential differences between breast tumor cell lines and primary tumors have recently been extended[6] to include mutations of tumor suppressor genes, such as the *CDKN2* gene that encodes the cell cycle regulatory cyclin-dependent kinase-4 inhibitor p16, because it was shown that the mutation is present in various cell lines but not in patients' tumors. Thus, despite the volume of work that has accumulated based upon the use of human breast cancer cell lines, it is clear that we cannot depend solely upon these cell lines for the best understanding of the cellular or molecular factors that regulate breast cancer progression in patients. Studies have described the isolation of cell lines from primary tumors, yet these systems are still few in number.[1,2,7,8]

In an alternative approach to the study of human breast cancer progression, investigators have examined the growth of patients' breast carcinomas directly implanted as xenografts into a variety of animal model systems, including the anterior chamber of the eye of guineapigs, lethally irradiated or thymectomized mice, and nude and severe combined immunodeficient (SCID) mice.[4,9-15] These approaches have also yielded a disappointingly low percentage of breast tumor growth compared to growth obtained with other major cancers of epithelial origin. In a comprehensive study that evaluated the growth potential of 433 human breast carcinoma specimens heterotransplanted into nude mice, Giovanella et al, reported that only 6.1% could be grown.[15] Two groups have analyzed the growth of human breast carcinoma implanted orthotopically (i.e., into the mammary fat pad) and both reported success.[16,17] However, in each study, a malignant specimen from only one patient was tested; thus, it is not possible to determine whether these results could be translated into a higher percentage of successful implants in a larger study. In the second of these studies, similar growth was obtained if pieces of the patient's tumor were placed subcutaneously or orthotopically; however, metastatic spread to the lung occurred in animals implanted orthotopically, an event that previously had not been observed.[17]

We have evaluated various protocols for improving the chances of obtaining growth and metastatic spread of human breast tumors by using SCID mice.[18,19] We report our findings of an evaluation of 48 human breast carcinomas implanted within the large, easily accessible abdominal (gonadal) fat pad (GFP) of SCID mice. The analysis reveals clear differences in the growth, histology, and metastatic potential among a variety of breast cancer patients' tumors, consistent with the clinical picture for this disease. The data obtained also indicate that use of this protocol can often result in the rapid and sustained growth, passage and metastatic spread of the implanted tumors. Factors that may affect rapid growth are discussed.

## ■ MATERIALS AND METHODS

***Animals.*** The CB17-scid/scid mice used for this study were either purchased from Taconic Laboratories (Germantown, Penn) or were produced in the breeding colony at Roswell Park Cancer Institute. They were housed in microfilter cages (Lab Products, Maywood, NJ). All cages, water and mouse diet (Teklad Mills, Wienfield, NJ) were supplied after being autoclaved. The cages were maintained inside an air-conditioned and light-controlled (12 h/day) room. All surgical procedures were performed in a laminar flow hood. Female mice were used for this study. Except for certain experiments, each mouse was implanted with an estrogen pellet (1.7 mg 17-∃-estradiol, 2 mg cholesterol, Innovative Research of America) at the time of initial surgery and at the time of passage. Both virgin and retired breeder mice were used for this study, as no obvious differences were observed.

***Tumor implantation.*** Fresh specimens of confirmed human breast malignancies (selected for this study only if the available specimen was large enough for implantation into at least two mice) were delivered to the laboratory shortly after their surgical excision. The tumors were sterilely minced with scissors (in RPMI 1640 containing 10% fetal bovine serum) to 1 to 2 mm sized pieces just prior to their implantation into subcutaneous sites and/or the gonadal fat pad of SCID mice. Mice were anesthetized via intraperitoneal injection of 0.35 to 0.45 mL of Avertin (2,2,2-Tribromoethanol, 12.5 mg/ mL) and placed on an operative metal cross. Typically, a 3-mm vertical incision was made on the right lower abdomen with scissors and the right portion of the gonadal fat pad was pulled up through the incision. One piece of tumor was implanted in the GFP by wrapping the GFP around the tumor and fixing it in place with 6-0 Dexon. The tumor+GFP was then replaced into the abdomen and the peritoneum was sutured. Skin clips were used to close the incision. At the time of surgery an estrogen pellet was placed subcutaneously on the back of the neck. After surgery, the mice were warmed until they awoke and then placed back into the cage.

***Tumor growth and histology.*** Although tumor growth and volume could often be discerned by palpating the site of implantation, animals were killed at various time points and the tumor+fat pad was removed for histologic analysis or for passaging of the tumor

into other SCID mice. Because the number of mice implanted varied considerably, we did not have a uniform schedule of time points for histologic examination, but instead tried to equally space the time points for killing mice over at least 6 months to 1 year; longer time points were achieved (14 months). If mice died naturally during the course of study, the fat pads were removed as quickly as possible after the time of death and processed for histologic evaluation. For histology, excised tissues were fixed for 24 hours in 10% buffered formalin. The tissues were dehydrated for 18 hours by an automatic tissue processor (in an Autotechnicon) and then were embedded in paraffin blocks. Five micrometer paraffin tissue sections were obtained by using a microtome. The tissue sections were stained with hematoxylin and eosin.

***In situ hybridization.*** To confirm human cells within the tumors, a biotinylated probe that recognizes human alpha-satellite DNA (p17H8) was used to react with tumor cells on frozen sections of tumor specimens.[20] Vecastain ABC kit (Vector Laboratories, Burlingame, Calif) was used for color development.

***Analysis of keratin expression.*** Frozen sections of tumor specimens grown within the SCID mouse were stained for type II keratin using immunofluorescence labeled-AE-3 monoclonal antibody that is specific for an epitope found in type II keratin (kindly provided by Dr Bonnie Asch).

## ■ RESULTS

We evaluated a variety of protocols and sites within SCID and nude mice in the hope of increasing the growth potential of surgical specimens of human breast carcinomas. We were largely unsuccessful in either maintaining or growing any tumors (either disaggregated prior to injection, or as intact pieces) in subcutaneous sites in either nude or SCID mice, confirming the previous studies of others.[1,2,14,15] However, rare instances of tumor growth were observed if intact pieces of tumor were implanted within a mammary fat pad site in SCID mice. These preliminary efforts led us to conclude that implanting small cubes of intact tumor (as previously done by others[12]), rather than injecting disrupted cells, and finding a fat pad large enough to wrap completely around the intact segment of tumor were important factors for supporting more consistent growth. Therefore, pieces of patients' breast carcinomas were implanted within the large GFP found just under the peritoneum in the abdominal cavity. A total of 48 surgical specimens of human breast tumors were evaluated histologically at various times after implantation in this site (Table 1). Figure 1 shows the location of normal GFP and growth of one of the earliest human breast tumors implanted within this site. The histologic pattern of that particular tumor (7443; see Table 1) is also shown. However, we quickly noticed that there was extensive variability with regard to growth potential and growth rates among the various surgical specimens. In many cases, tumors exhibited little or no palpable growth for several months and then would begin to exhibit steady, yet remarkably slow, growth. The growth of some xenografts might have been missed altogether if external palpation were the only means used to assess growth; the abdominal location of these implanted xenografts makes palpation for their presence and growth more difficult until they have at least doubled or tripled in size. Often, growth could only be discerned via histologic analysis or by visual inspection of the isolated fat pad following dissection. In many cases, tumors would remain small for variable periods of time (1–5 months) and then suddenly exhibit fast growth; others would grow quickly from the first week after implantation. Moreover, we have successfully passaged most of the tumors with fast growth rates at least three times, several of the older tumors as many as 10 times, and currently continue to passage these tumors. It is also significant that within the group of mice implanted with pieces of the same surgical specimen there is varied success of tumor growth (i.e., for any given tumor, we observed that there were often one or more mice that did not sustain any growth, whereas mice implanted with a different piece of the same tumor may exhibit rapid growth).

We conducted a histologic evaluation of the engrafted tumors listed in Table 1. In addition to considerable variation in the rate of growth exhibited by various tumor xenografts, we also noted significant variation in the histology and vascularization of various tumors. The histologic patterns of the fast-growth tumors could vary considerably and, in addition to the tumor shown in Figure 1C, three additional patterns we observed are shown in Figure 2. Some tumors grow in rather diffuse patterns, with little or no overall organizational state (Figs. 1C and 2A); these tumors were often seen to be well vascularized, presumably from the blood vessels of the GFP. There appears to be no obvious encapsulation of these tumors, and the individual cancer cells appear to invade the interstitial spaces between adipocytes of the fat pad. However, distinctive differences in the histologic pattern of two other fast-growth tumors are shown in Figure 2. In one case (Fig. 2B), the tumor consistently grew as individual foci that became necrotic in the center, whereas in another case (Fig. 2C) the tumor grew as individual, elongated cords that did not become necrotic in the interior. Similarly, the histologic patterns seen when slow-growth tumors were analyzed varied considerably (Fig. 3B–D). Although it is our impression that obvious vascularization is not as frequent in tumors that grow slowly as in faster growing tumors, this conclusion has not been verified experimentally.

When many slow-growing and fast-growing tumors were analyzed at various times after implantation, certain common features became evident. Tumors that were excised 1 to 3 months after implantation often appeared similar to the tumor shown in Figure 3A; much of the interior of the original implant (whose borders can be seen adjacent to the adipocytes of the fat pad) was acellu-

**Table 1. Pathologic Diagnosis and Tumor Growth in SCID Mice**

| Tumor # | Pathologic Diagnosis | Nodal Involvement | Estrogen Receptor ≥ 15 fmol/mg | Growth in SCID Mice[a] |
|---|---|---|---|---|
| 7326P | ILC | - | + | 0/2 |
| 7356[c]P | IDC | + | - | 2/4 |
| 7418[c]P | IDC | - | - | 1/2 |
| 7421P | Intraductal Ca (with microinvasion) | - | - | 1/2 |
| 7443[b,c]R | IDC, recurrence after chemotherapy, RT | N.A. | - | 4/4[d] |
| 7486[b]P | IDC | - | - | 5/5[d] |
| 7526P | IDC | - | - | 2/5 |
| 7528P,CT | IDC, prior chemotherapy for lung cancer | - | - | 3/4 |
| 7543P | IDC | + | + | 3/5 |
| 7553P | IDC | - | + | 2/4 |
| 7556[c]P | Papillary cystic Ca | - | + | 3/5[d] |
| 7573P | IDC | - | + | 0/5 |
| 7582P | ILC | + | + | 4/5 |
| 7595P | IDC, matched with 7744 | + | - | 0/3 |
| 7608[c]P | IDC | + | - | 2/2 |
| 7628R | IDC, recurrence after chemotherapy, RT | N.A. | - | 5/5[d] |
| 7655P | IDC | + | + | 1/3 |
| 7661P | IDC | + | + | 0/8 |
| 7679P | IDC | + | - | 5/9 |
| 7717P,A | IDC, primary after chemotherapy | + | + | 2/2[d] |
| 7722[b]LN | IDC, regional nodal metastasis | N.A. | - | 4/4[d] |
| 7744P,A | IDC, primary after chemotherapy | + | - | 6/10 |
| 7748P | ILC | + | + | 4/4 |
| 7764[b,c]P,A | IDC, primary after chemotherapy | + | - | 9/10[d] |
| 7784P | ILC | + | + | 2/2 |
| 7788P | IDC | + | + | 5/5 |
| 7806LN, CT, RT | IDC, LN recurrence, after chemotherapy, RT | + | + | 0/3 |
| 7821P | IDC | + | + | 0/4 |
| 7841P | IDC | - | + | 0/2 |
| 7842P | IDC | + | + | 3/3 |
| 7857P | IDC | - | + | 0/3 |
| 7869[c]P | IDC | + | + | 6/8 |
| 7882P | IDC | + | + | 2/2 |
| 7892P | IDC, mucinous | N.A. | + | 0/3 |
| 7897P | IDC | + | + | 1/3 |
| 7914[b]P,CT | IDC, prior opposite BRCA + chemotherapy | + | + | 4/5[d] |
| 7929[b]P | IDC | - | - | 4/5[d] |
| 7960P | IDC | + | - | 0/2 |
| 7967P | IDC | + | + | 2/9 |
| 8001P | IDC | + | + | 1/2 |
| 8029[b,c]P | IDC | + | - | 2/5[d] |
| 8038P | IDC | N.A. | - | 4/5[d] |
| 8070P | IDC, atypical medullary | + | - | 1/2 |
| 8099[b]P,A | IDC, primary after chemotherapy | + | - | 5/5[d] |
| 8180P | IDC | + | + | 3/5 |
| 8194P | IDC, matched with 8419 | + | + | 0/2 |
| 8200LN,A | IDC | + | N.A. | 3/5 |
| 8419LA | IDC | + | + | 1/3 |

[a] Numbers refer to the animals in which growth by histologic evaluation was obtained in the first passage out of the total number implanted with pieces of the original surgical biopsy material.
[b] Showed evidence of metastasis to other organs in later passages.
[c] Tumors that expressed Type II keratin and were demonstrated to be human by in situ hybridization using a human DNA specific probe.
[d] Showed evidence of exceptionally fast growth, and were passaged multiple times.
Abbreviations: P, primary (no prior treatment); A, neoadjuvant chemotherapy (chemotherapy prior to tumor removal); LN, lymph noide; R, recurrence; N.A., not available because not done or done elsewhere prior to this study; RT, radiation therapy; CT, previous chemotherapy; ILC, invasive lobular cancer; IDC, invasive ductal cancer.

lar, and consisted of nothing but connective tissue (collagen), while most of the tumor cells were found on the periphery of the implant, adjacent to the fat pad cells. Whether the cells in the interior had died, or whether they migrated to the periphery in response to other stimuli is not known. In most of the slow-growing and fast-growing tumors that were evaluated, an eventual asymmetrical outgrowth into the GFP occurred (i.e., the tumor cells appeared to "spill out" from one region of the implant; Fig. 2B & C). The implant itself became nearly devoid of cells. The timing of this event is variable, and appears to be dictated by properties of each tumor.

Some tumors were found in a state similar to that shown in Figure 2A for as long as 6 months, whereas others appeared to progress to the stages depicted in Figure 3B & C within several months and then continued to grow at a very slow but inexorable rate. Often a SCID mouse bearing a particular tumor would die of other causes and the tumor would be removed and found in the stages shown in Figure 3B & C. We also found occasional examples where a relatively uniform outgrowth of new tumor from the collagen center, which is devoid of cells, had occurred (Fig. 3D). This small but healthy tumor was found after 14 months of remarkably slow growth in the SCID mouse. Therefore, it is apparent that if histologic analysis is not performed, a significant fraction of slow-growing tumors may go undetected. However, this caveat does not apply to the inability to detect subcutaneous growth in SCID or nude mice, where the ability to palpate tumors implanted subcutaneously is considerably more accurate.

The histologic pattern of each tumor was maintained through several passages in the SCID mouse (at this time over seven passages for the tumors shown in Fig. 2A & B and three passages for the tumor shown in Fig. 2C). Moreover, the pattern evidenced by the tumor shown in Figure 2B was found to be reproduced in metastatic sites within the SCID mouse (Fig. 4).

Occasionally, we found zones of apparently normal mammary epithelial cells growing as isolated duct-like structures within the GFP, or occurring between zones of tumor (Fig. 3E). These cells may have derived from normal cells still present in the biopsy or may represent early malignant cells that do not as yet reflect the phenotype of other invasive cells in the tumor. The histologic criterion indicating that there was no growth of tumor in a particular mouse is shown in Figure 3F. The original explant can be seen within the GFP, is composed of connective tissue only, often degraded in many sites, but with no evidence of tumor cells. In this study, 23% of the patients' tumors failed to grow in any of the implanted animals (Table 1).

Quite relevant to the clinical issues related to human breast cancer, many of the faster-growing tumors also exhibited eventual metastatic spread within the SCID mouse (Fig. 4 and Table 1); metastatic foci of tumor were observed by us in the liver, lung, diaphragm, adrenals, abdominal wall, and subcutis, in decreasing order of frequency. Bone marrow was not analyzed in this study, but its involvement is currently being investigated. With one exception (8029), these tumors did not exhibit metastases within the first passage in the SCID mouse. Metastatic spread was often not observed for many months or until the xenograft had undergone several passages (1–5 passages was the range in our analysis). Again with one exception (7722), the tumors that showed metastatic spread within the SCID mouse were derived from primary or recurrent chest wall breast tumors in patients with no clinical signs of distant metastasis. Although nodal involvement was seen in most


**Figure 1** (A) Normal GFP of a 2-month-old SCID mouse showing its symmetrical positioning in the abdomen (arrows). (B) Arrowhead indicates a tumor seen 7 weeks after implantation of a surgical specimen (7486). (C) Histology of the tumor shown above indicates dense tumor growth, with nonencapsulated tumor cells invading the spaces between the surrounding adipocytes (arrow). Mitotic cells (arrowhead) are seen frequently. (Bar = 20 μm)

(but not all) fast-growing cases, this does not appear to be a critical factor for predicting growth rate or metastatic potential because nearly all of the patients whose tumors failed to grow whatsoever were also node positive (Table 1).

As with growth of patients' tumors in the fat pad, the metastatic tumors exhibited remarkable heterogeneity of growth patterns. They differed in which organ was most often involved (e.g., lung vs. liver) and also in the histology of the metastatic growth. Within the lung, a tumor may form one or two large foci of relatively undifferentiated tumor growth that are visible to the eye (Fig. 4A) or may grow as numerous, smaller micrometastases that are not visible upon gross inspection (Fig. 4B). Within the liver, we have observed foci of tumor that grow as nodules that are virtually identical to those seen in the tumor originally implanted into the GFP (Compare Fig. 4C with 2B, which are of the same tumor). Alternatively, liver metastasis can appear

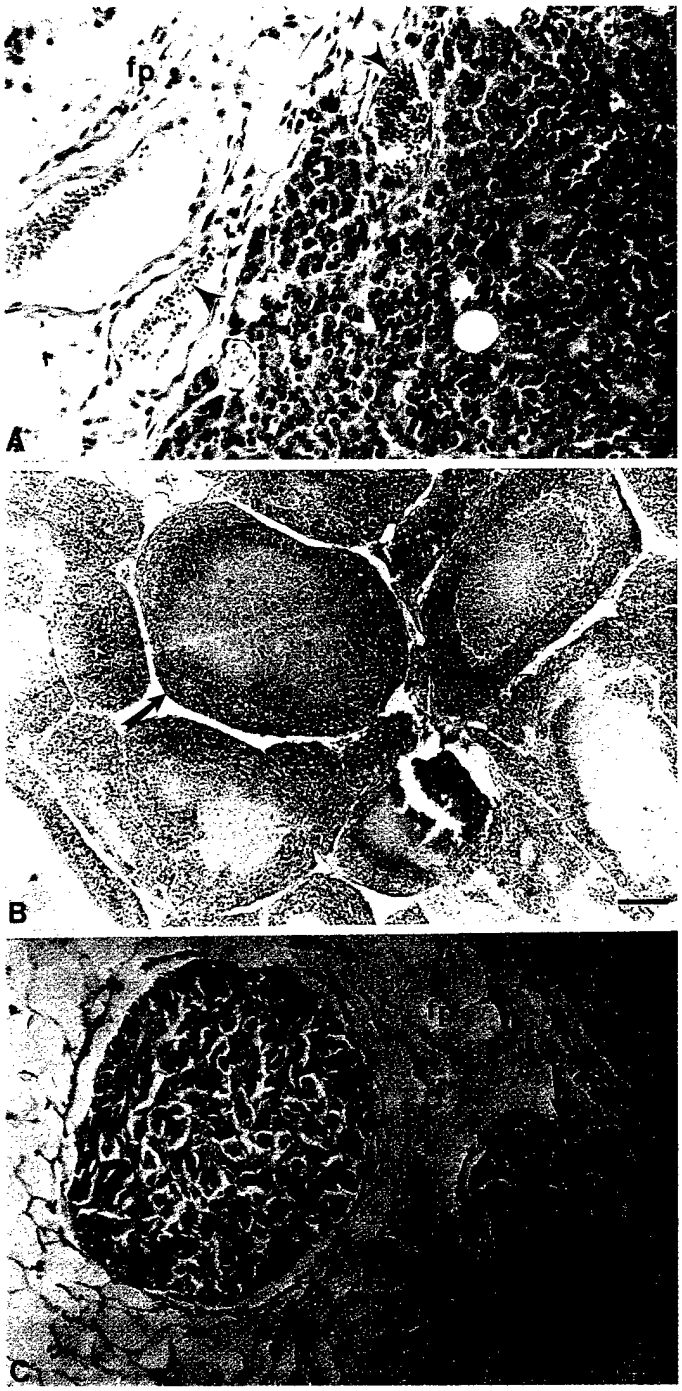


**Figure 2** (A) This tumor (7743) exhibited a diffuse growth pattern similar to that seen in Fig. 1C, except that vascularization is more obvious (arrowheads). (B) In this example, (7722) the tumor grew in discrete foci that appeared necrotic in the center. Active mitotic growth is seen in the rim of tumor surrounding the necrotic tumor and an identical pattern was seen with this same tumor in metastatic sites (compare with Fig. 4C). (C) This tumor (7556) grew as elongated cords that intermeshed throughout the GFP. Unlike the tumor in Fig. A, the centers of these cords of cells did not usually appear necrotic. (Bar = 25 μm in A,C; 40 μm in B)

as a multiple large foci of tumor growth that appear sinuous, with connective tissue spaces evident, and without obvious necrosis.

In this study, we observed growth of both estrogen-receptor (ER) positive and negative tumors. During passaging of ER-positive tumors (four tumors), we observed that these tumors require estrogen supplements at the time of surgery to survive and grow. ER-negative tumors however, grew in the absence of added estrogen (data not shown; see also ref. 13).

In this study, human origin of tumor cells growing in the GFP was confirmed by an in situ hybridization analysis and using the anti-human keratin antibody, AE-3 (data not shown; the tumors analyzed are indicated in Table 1).

To determine if there were any factors useful in predicting whether a given patient's tumor would grow quickly, slowly, or not at all in SCID mice, we evaluated various available clinical data, including node status, ER expression, and whether or not the patients had previous treatment (e.g., chemotherapy) prior to surgical removal of the tumor.

We observed that of the 36 primary, untreated cancers transplanted (Table 1), 5 (14%) grew rapidly, 21 (58%) grew slowly, and 10 (29%) failed to grow. In comparison with six primary tumors from patients who had received chemotherapy previously (Table 1), four (67%) were rapid growers, two (33%) were slow growers, and none failed to grow ($P = 0.011$, Chi-square). Excluding metastatic lymph node xenografts, being ER-negative predicted rapid growth (8 of 19 [42%] ER-negative tumors grew fast while only 3 of 25 [12%] of ER-positive tumors did so; $P = 0.035$; Fisher exact test). The presence of axillary nodal metastasis was not predictive (5 of 28 node-positive tumors [18%] grew fast and 3 of 12 node-negative tumors [25%] did so; $P = 0.677$, Fisher exact test).

## ■ DISCUSSION

Experimental research on human breast cancer and the identification of better markers of tumor progression and risk assessment has been hampered by the lack of an animal model in which the growth of surgical specimens of primary recurrent breast carcinomas can be studied. Such an animal model could be used not only to study the cellular and molecular properties of various patients' breast carcinoma specimens, but to evaluate preclinically the effect of various chemotherapeutic agents, immunotherapies, or various environmental factors such as diet.

Most experimental models of breast cancer involve the use of rodent mammary tumors; these include models of spontaneous mammary tumor development, chemically and virally induced mammary carcinomas, and transgenic mice.[3] With regard to animal models of human breast cancer, the predominant model depends upon the use of a few well-studied cell lines that grow as solid tumors when inoculated into immunodeficient mice.[1-3] Although the breast tumor xenografts derived from cell lines are advantageous for many types of studies (primarily because the tumors are of human origin), relatively few human cell lines have been developed for

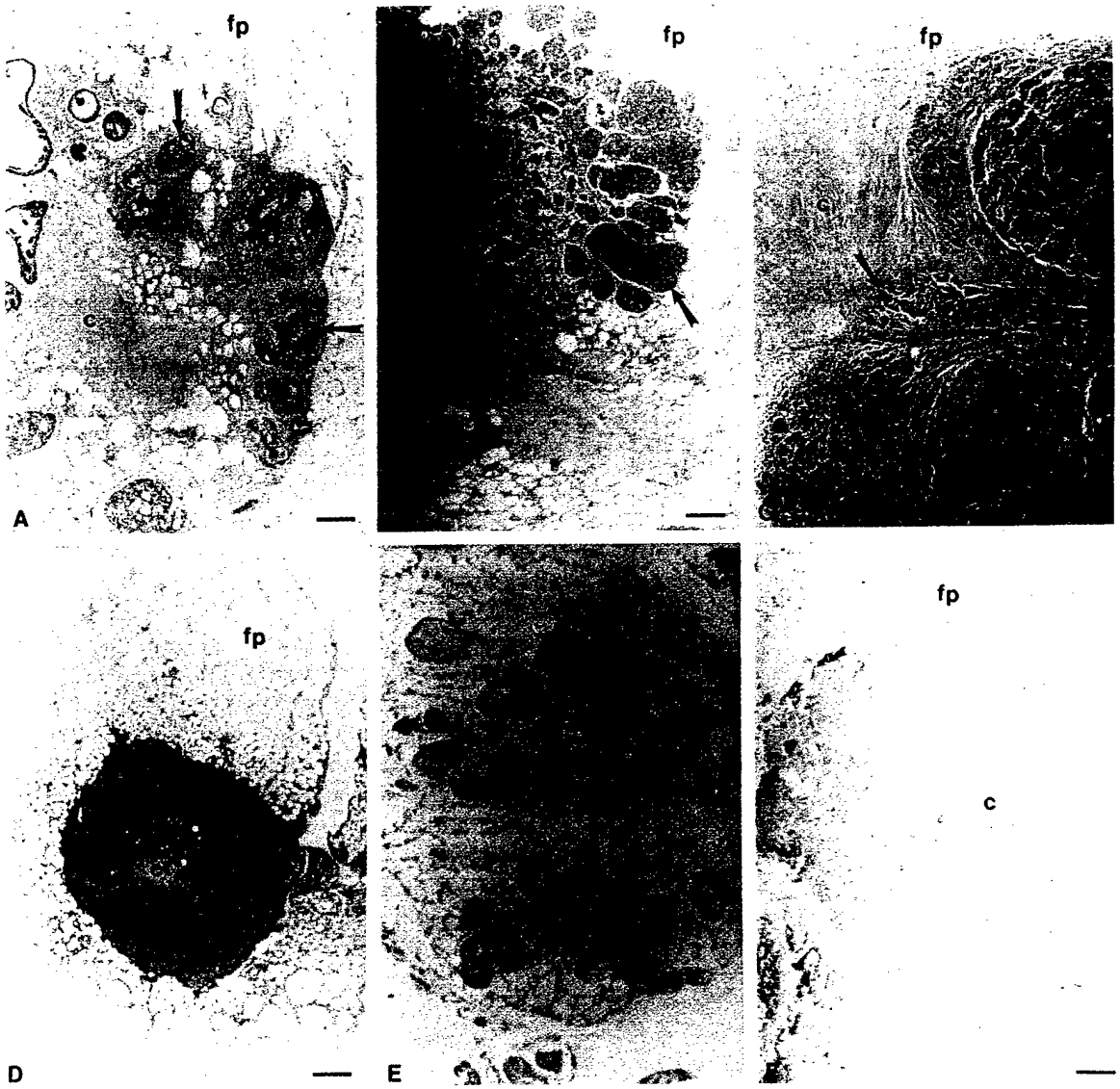


**Figure 3** (A) Early pattern of tumor growth (7914) reveals numerous clusters of tumor cells at the periphery of the explant, but no tumor cells in the center of the collagen. (B, C) Representative slow-growth tumors exhibiting tumor growth from one region of the explant ( 7748 and 8180, resp). Again, there is little tumor remaining in the original explant. (D) Another example of slow-growth tumor (7869) in which tumor cells completely surround the region of the explant. (E) An example of apparently normal duct-like growth that occurs often in the various regions of the GFP implanted with breast tumor tissue (from 8180). (F) Representative histology of a tumor that did not grow after 3 months of engraftment. Only the original collagenous region of the implant remains, with little or no tumor cells visible. (c, collagen; fp, fat pad) (Bar = 60 μm in A, B, F; 40 μm in C; 75 μm in D)

this purpose, and most of these were derived from highly metastatic cells from effusions. It is clear from the abundance of studies using these human breast tumor cell lines that the factors that regulate the proliferation of these cells in vitro are significantly different from those that regulate normal human mammary epithelium,[2] and may also differ considerably from those that regulate tumor development in vivo. Thus, to acquire a thorough understanding of the biologic effects of the genetic alterations thought to be involved in breast cancer progression, it is essential to continue development and improvement of the existing experimental models, making it possible to isolate and study cells from various tumors on a more routine basis.

The histologic analyses presented in this paper indicate that placing intact pieces of surgical specimens of primary human breast carcinomas within the confines of the large SCID mouse GFP can often result in their sustained growth; for many tumors, the growth rate is fast enough to result in large amounts of tumor tissue that can be passaged into additional mice within weeks to months. For most of these tumors, metastatic spread within the mouse also occurred. The majority of tumors implanted exhibited a slower growth rate, with xenografts only doubling or tripling in size over a period of 6 months to 1 year or more.

The GFP was initially tested since it was a large, easily accessible fat pad that was histologically similar to

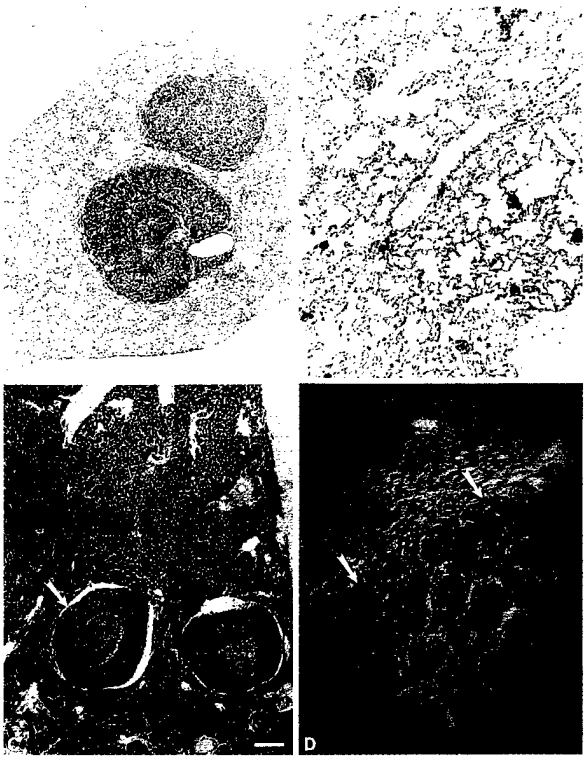


**Figure 4** (A) Large foci of tumor growth are present within the lung parenchyma. This metastasis occurred during the 3rd passage of tumor specimen 7443. (B) Another example of lung metastasis: scattered small foci of tumor growth can be seen throughout the lung parenchyma. (C) Liver metastasis seen with tumor 7722. Note the similarity of metastatic tumor growth to that seen in this same tumor growing within the GFP (Fig. 1B). (D) Liver metastasis of tumor 8029 reveals a different histologic organization from that seen in Fig. C (arrows indicate regions of tumor growth seen in each case). (Bar = 40 μm)

the mammary fat pad, a site in rodents that has long been used by breast cancer biologists. However, unlike the mammary fat pad, the GFP is large enough to wrap completely about an intact segment of tumor, a factor we believe to be important for more successful tumor growth. Importantly, the GFP and the omentum are frequently the sites of metastasis of several types of cancers,[21] suggesting that this site may be rich in factors that sustain the growth of tumors. The large size and ease of accessibility of the GFP compared with the mammary fat pad would make this site preferable for various types of studies. A full comparison of both sites is warranted and is underway in our laboratory. The GFP of mice appears to be highly vascularized and consists largely of white fat cells (adipocytes) mixed with regions of brown fat and is similar in histology to the mammary fat pad, except that it is completely devoid of mammary epithelium; further, in SCID mice few other connective tissue cells were seen whereas in normal (Balb/c) mice, frequent macrophages and lymphoid cells were seen among the adipocytes throughout the tissue (E. Repasky, unpublished data). Many studies have been conducted on the metabolic importance of this organ in various rodents.[22,23] The interior of the gonadal fat pad may provide a particular microenvironment that stimulates or maintains breast tumor growth. Indeed, normal and malignant breast tissue develop near, and are maintained in close association with, fat cells in the mammary fat pad. Noteworthy in this regard is the fact that fat tissue can synthesize estrogen or harbor other factors that stimulate breast epithelium.[24-26] However, in this study we observed an estrogen-dependence of tumors that were ER-positive, whereas ER-negative tumors grew in the absence of estrogen supplementation. In the case of at least one ER negative tumor however, we have determined that it will grow more rapidly if estrogen is provided to the animal (R. Burd, Y. Xu, and E. Repasky, unpublished data).

The impaired immune status of the SCID mouse (lacking both T and B cells) mouse may also play an important role in enhancing tumor growth in the GFP, since preliminary data indicate that the SCID mouse GFP sustains growth better than a similar implantation in the nude mouse (T. Sakakibara and E. Repasky, unpublished data). Several reports indicate that various other tumors may grow better in the SCID mouse than in the nude mouse (see review in ref. 27). This may be due to the absence of both T and B cells within the SCID mouse, or to the lack of circulating xenoreactive antibodies that may interfere with breast tumor growth. SCID mice do however have an active natural immunity (including natural killer cells), and at this time, we do not know if the growth of breast xenografts can be enhanced by using NOD/LtSz-scid mice, in which the natural killer cell activity is significantly reduced.[28] However, preliminary data has shown increased growth potential in mice treated with anti-asialogmi, which blocks NK cell activity (R. Burd, S. Dziedzic, E. Repasky, unpublished data).

Occurrence of metastatic spread of tumors derived from some patients and not others may have important clinical implications; a longer term analysis of the clinical outcome of the patients used in this study will help to verify this hypothesis. Similarly, expression of various pathohistologic growth patterns of the xenografts as well as the differences in growth rate indicated by this study may directly reflect the different biologic properties and malignant potential of the original tumor specimens. That there is a heterogeneity of cell types within primary or locally recurrent tumors is exemplified by the fact that within the xenograft we sometimes found regions of cells that were similar in appearence to those found in normal mammary epithelium, sometimes in close proximity to obviously more invasive cells. We observed this normal growth pattern most often in association with the slow-growing tumors. Exactly how normal these cells are is open to question. But it is of interest to note that benign human breast tumors have been reported to grow in nude mice[13] and, in fact, were reported to grow more consistently than malignant specimens. It has also been noted that, although invasive cells from primary breast tumors are difficult to culture, cells

from benign lesions can be readily cultured.[29]

In this study, we analyzed several clinical factors that may be predictive of whether a tumor grows quickly or not at all. We found that a high proportion of tumors that grow quickly are ER-negative and derived from patients who had received previous chemotherapy. Since previous reports on the growth of patients' breast tumors in nude and SCID mice have not included this type of additional clinical data, we cannot compare our results or determine if these factors may play a role in the enhanced growth potential observed in this study. It is important to note that even with chemotherapy or the absence of estrogen receptors, tumors would still often fail to grow if implanted subcutaneously, so there appears to be a growth advantage for even these tumors if implanted within the gonadal fat pad. In one case, we derived primary untreated tumor and primary tumor following chemotherapy from the same patient (compare tumor 7595 with 7744). Although this patient's untreated primary tumor failed to grow in 3 mice, her chemotherapy-treated primary tumor exhibited slow growth in 6 out of 10 mice. These results suggest that future reports should include additional clinical data with rgard to patient specimens that are used, since these factors may play a role in whether a tumor grows quickly, slowly, or not at all.

Numerous questions are raised by this study, such as the possible role of vascularization in regulating whether a tumor grows quickly, slowly, or not at all, or what the relationship is (if any) exists between prior chemotherapy, estrogen-receptor status, and growth rate of the xenograft. We are currently conducting an immunocytochemistry analysis of cell surface markers of clinical interest, such as HER-2/neu and p-glycoprotein expression, among the various xenografts using paraffin-embedded material that has been prepared from each. These and other data may be useful in learning more about the factors that regulate the growth rate and metastatic potential of the various xenografts. However, we believe that the use of the SCID mouse gonadal fat pad model, as described here, can provide an easily accessible site for growing and passaging many human breast carcinomas. The ability to collect large amounts of tumor tissue from various passages and from metastatic sites in this model will be useful for genetic, biochemical, and immunologic analyses of breast tumor cells; moreover, the ability to subpassage human breast tumors into large numbers of mice will make possible a variety of experimental studies to evaluate new therapeutic approaches for this disease.

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# Tumor Cell Apoptosis, Lymphocyte Recruitment and Tumor Vascular Changes Are Induced by Low Temperature, Long Duration Whole Body Hyperthermia

RANDY BURD,[1] THOMAS S. DZIEDZIC,[1] YAN XU,[1] MICHAEL A. CALIGIURI,[2] JOHN R. SUBJECK,[3] AND ELIZABETH A. REPASKY[1]*

[1]Department of ~~Molecular~~ Immunology, ~~Roswell Park Cancer Institute, Buffalo, New York~~

[2]Department of Medicine, ~~Roswell Park Cancer Institute, Buffalo, New York~~ AND

[3]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York

A single treatment of low-temperature, long-duration, whole-body hyperthermia of mice bearing either human breast tumors or transplantable syngeneic murine tumors for 6–8 hr can cause a temporary reduction of tumor volume and/or a growth delay. In both animal model systems, this inhibition is correlated with the appearance of large numbers of apoptotic tumor cells. Because this type of mild heat exposure, comparable to a common fever, is not itself directly cytotoxic, other explanations for the observed tumor cell death were considered. Our data support the hypothesis that this hyperthermia protocol stimulates some component(s) of the immune response, which results in increased antitumor activity. In support of this hypothesis, increased numbers of lymphocytelike cells, macrophages, and granulocytes are observed in the tumor vasculature and in the tumor stroma immediately following this mild hyperthermia exposure. In Balb/c mice, an infiltrate persists for at least 2 weeks. Using the SCID mouse/human tumor system, we found that both host natural killer (NK) cells and injected human NK cells were increased at the site of tumor following hyperthermia treatment. Experiments using asialo-GM1 antibodies indicate that the tumor cell apoptosis seen in the SCID mouse appears to be due ~~almost entirely~~ to the activity of ~~at least~~ NK cells, although additional roles for other immunoeffector cells and cytokines appear likely in the immunologically complete Balb/c model. Another interrelated hypothesis is that immunoeffector cells may have greater access to the interior of the tumor because we have observed that this treatment causes an obvious expansion in the diameter of blood vessels within the tumor and an increase in nucleated blood cells within the vessels, which persists as long as 2 weeks after treatment. Further study of the mechanisms by which mild hyperthermia exerts antitumor activity could result in this treatment protocol being used as an effective, nontoxic adjuvant to immunotherapy and/or other cancer therapies. J. Cell. Physiol. 176:00–00, 1998. © 1998 Wiley-Liss, Inc.

There are compelling biological reasons that continue to drive a century-long interest in the use of hyperthermia in the treatment of human cancer (Hahn, 1982; Oleson et al., 1988). The origins of hyperthermia as a cancer treatment modality are linked with some of the earliest immunotherapy literature because it has been recognized that the bacterial toxins given to cancer patients to stimulate their immune system also give rise to high fevers; subsequently, it was observed that those who developed the highest fevers were most often the ones who had the longest survival times (Nauts and McLaren, 1990). In the early literature, it was also noted that individuals who survived infectious disease associated with fever may exhibit tumor regression (e.g., Busch, 1866; Westermark, 1927). However, modern clinical hyperthermia efforts to treat human cancers do not use fever as the source of heat but have instead focused primarily on the use of externally applied heat (usually by using microwaves) directed at only a limited region of the body for a short duration. Although the physics behind delivery of effective "local hyperthermia" to tumors is complex, many years of experimental study (Oleson et al., 1988) have led to the

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*Correspondence to: Elizabeth A. Repasky, Ph.D., Department of ~~Molecular~~ Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263. E-mail: repasky@sc3101.med.buffalo.edu

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initiation of several clinical trials in the mid-1980s that met with contradictory results. However, several more recent prospective randomized trials have now plainly demonstrated the significant potential of local hyperthermia to improve antitumor effects when combined with either chemotherapy or radiation for several different types of human cancers (Seachrist, 1993; Valdagni and Amichetti, 1993; Sugimachi et al., 1995; Overgaard et al., 1996; Vernon et al., 1996).

Although local hyperthermia was initially envisioned as a type of thermal radiation therapy in which a heat dose hot enough to kill cancer cells directly would be selectively delivered to the tumor mass, the tumor temperatures actually achieved during treatment are often well below those required for any direct cytotoxic effect (Oleson, 1995). Therefore, the clinical benefit of local hyperthermia may more likely be the result of the low heat temperatures achieved, thereby indirectly improving radiation response by increasing tumor oxygenation via increasing blood flow into the tumor. Studies from several other groups have focused specifically on radiobiological effects of "low-temperature" local hyperthermia, in particular the effects of low (heating) temperature on radiosensitization and reoxygenation and alterations in blood flow (e.g., Armour et al., 1991, 1993; Hetzel et al., 1992; Horsman and Overgaard, 1996; Song et al., 1996a,b).

Whereas local and often regional hyperthermia have been the primary means of applying high-temperature hyperthermia, studies over many years have also investigated the effectiveness of whole-body heating (e.g., Robins et al., 1993). In this procedure, the core temperature of the animal or patient is usually raised to a very high temperature range (approximately 41.8–42°C) that can be sustained for a only a short time (30 min to 2 hr; Pettigrew et al., 1974; Bull, 1983). The conventional (high temperature, short duration) thermal protocol for whole-body hyperthermia (WBH) in combination with various chemotherapeutic drugs results in enhanced cytotoxicity of both tumor and normal tissue, which seriously reduces therapeutic gain (Gerad et al., 1983; Wondergem et al.,, 1988; Ohno et al., 1991). However a novel low temperature (i.e., mild hyperthermia) protocol, which can be applied for much longer periods of time, was found to significantly improve antitumor effects without added normal tissue toxicity from carboplatin and tumor necrosis factor (Sakaguchi et al., 1994). Surprisingly, when the low-temperature, long-duration WBH procedure was applied alone using a rat tumor model, without any other additional therapy, a tumor growth delay was still observed together with a significantly (50%) reduced incidence of lymph node metastases (Matsuda et al., 1997).

One of the most important biological questions arising from these recent studies concerns the mechanism by which such a mild systemic hyperthermia treatment applied for a long duration produces such significant, albeit temporary, antitumor responses. Recent studies from our laboratory have indicated that a nearly identical low-temperature, long-duration whole-body heat exposure in mice leads to several important structural and functional changes in lymphocytes, suggesting that this treatment may be able to stimulate the activity of various immune effector cells (Di et al., 1997). We present several studies on the effects of low-temperature, long-duration WBH on the growth and histological appearance of human and murine tumors. Our studies reveal that a mild heating of animals bearing tumors for a period of several hours results in a significant lymphoid infiltrate and tumor cell apoptosis due to (at least) the activity of natural killer (NK) lymphocytes. These effects are seen with a simultaneous expansion of the volume of tumor vasculature, which could be useful for enhancing the targeting of immunoeffector cells and the delivery of reagents into the tumor interior. Although the mechanism(s) of these responses to elevated body temperature induced by external means is not yet known, it is highly likely that we are exploiting a heat-sensitive physiological response already in place that helps stimulate immunoeffector activity during a fever.

## MATERIALS AND METHODS

### Tumor implantation/growth

For some experiments, specimens of human breast tumors were embedded into the abdominal gonadal fat pad of SCID mice (Sakakibara et al., 1996) and allowed to grow for 3 weeks before receiving hyperthermia treatment. For other experiments in which regular tumor volume measurements were taken, human breast tumors that had been passaged several times in SCID mice were dissected into small pieces (2 mm in diameter) and implanted subcutaneously into SCID mice. Murine colon 26 tumors were injected subcutaneously into Balb/c mice from a single cell suspension ($1 \times 10^6$ cells/ml). WBH was initiated after the tumors reached a volume of approximately 60 $mm^3$. The tumors were measured with a venier caliper every 2 days to determine the shortest diameter (A) and the longest diameter (B). The volume was then calculated by using the formula $V = (A^2B)/2$. The relative tumor growth ($T_r$) was then calculated for each tumor by dividing the volume of the tumor at any given day after WBH ($T_d$) by the volume at the start of WBH ($T_0$). Data were analyzed for statistical significance by using Student's t-test. A value of $P < 0.05$ was considered statistically significant.

### Whole-body hyperthermia treatment

Mice were placed in microisolator cages preheated to 39°C that contained food, bedding, and water; it is important that water temperature be at least 39°C. The cages were then placed in a gravity convection oven with preheated incoming fresh air (Memmert model BE500, East Troy, WI). The average body temperature of the mice before WBH was 37.5°C. The temperature was then increased gradually (1°C) every 30 min until a core temperature of 39.8°C (±0.2°C) was achieved. The temperature of the incubator was then adjusted when necessary to maintain a constant core temperature of 39.8°C for 6–8 hr. During the heating, the core temperatures of the mice were monitored continuously by an inserted rectal thermocouple (prepared from fine wire) taped to the tail (Doric, Tredicator 412A; accuracy of ±0.1°C). The thermocouple and readout were calibrated against a National Bureau of Standards calibrated mercury thermometer before each experiment. In some experiments, the body temperature was monitored with the Electronic Laboratory Animal Monitoring System from Biomedic Data Systems (Maywood,

NJ). Microchip transponders 14 mm × 2.2 mm were subcutaneously implanted into the dorsal thoracic area with a trocar. Temperatures were collected using a scanner/readout. When compared with the core temperature readings using the thermocouple, the difference in temperature was never greater than ±0.1°C. We found that it was not difficult to maintain core temperatures between 39°C and 40°C. When heating several animals, internal variability between individual animals was ±0.2°C.

### Depletion of murine immune cells

To deplete NK cell activity, asialo GM1 antibody (Wako Chemicals, Richmond, VA) was diluted 1:5 in phosphate buffered saline (PBS) and 100 μl was injected i.p. 3 days prior to WBH and every 4–5 days thereafter. Technical information provided regarding this antibody indicates that there is a very low cross reactivity of the antibody with other cell types such as monocytes.

### Immunofluorescent staining

Tumors were removed from the gonadal fat pad, immediately immersed in Tissue Tek O.C.T. compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Sections (10 μm) were cut on a cryostat, placed on slides, and allowed to dry for 30 min at room temperature (RT). Slides were stored at −70°C until used. For immunofluorescent staining with asialo GM1 antibody, a two-layer technique was used (Parr et al., 1990). Slides were first air dried for 30 min RT or fixed 10 min in acetone. The slides were then incubated with 10% normal goat serum/PBS to block nonspecific binding. Rabbit anti-asialo GM1 antibody (1:250) was applied for 2 hr as the first layer. As a negative control, duplicate sections were incubated with 10% normal rabbit serum for 2 hr. Mouse spleens were used as a positive control. A FITC-conjugated goat anti-rabbit antibody (1:200) was applied for 1 hr as the second layer. Propidium iodide was used as a counterstain. Slides were viewed using a confocal microscope.

### Labeling human NK cells

Highly purified human NK cells ($5 \times 10^6$ cells/ml) were incubated with 2 μM DiI for 30 min on ice. The cells were washed three times in PBS, and then $2 \times 10^6$ cells were injected i.p. into mice bearing abdominally implanted human breast tumors at least 24 hr before hyperthermia. Immediately following treatment, the tumors were removed and frozen sections were prepared as described.

### Detection of apoptosis

Apoptosis was detected using the Oncor ApopTag Plus In Situ Apoptosis Detection Kit (Gaithersburg, MD). Briefly, tumors were removed 8–16 hr after WBH and fixed in 4% formalin for 14 hr. Paraffin sections were treated with proteinase K for 20 min. TdT enzyme was then added for 1 hr at 37°C to label fragmented DNA ends with digoxigenin nucleotides. Antidigoxigenin fluorescence antibody was then added for 30 min. A wet mount was prepared using propidium iodide, and the slides were observed using confocal microscopy.

## RESULTS

Two tumor models were analyzed in this study: (1) SCID mice implanted with human breast tumor xenografts (Sakakibara et al., 1996) and (2) Balb/c mice implanted with syngeneic tumors. When mice bearing abdominally implanted breast tumors were given low-temperature, long-duration WBH for 6 hr using the warm air environment technique and the tumors are excised and weighed 8 days later, they are found to be significantly lower in weight (Fig. 1A). The control group had an average weight of 0.94 g (±0.1 g), and the treated group had an average weight of 0.60 g (±0.08, $P < 0.05$). The mice themselves were not seen to differ in any other way; other internal organs appeared completely normal, except that the heated mice are often seen to have an enlarged spleen. Other experiments revealed little or no effect on tumor volume if the hyperthermia treatment was given for only 2 or 4 hr. However, if the heating was conducted in three intervals of 2 hr separated by two 15-min breaks at RT (so that the total time of heating was still 6 hr), we observed tumor volume reduction that was indistinguishable from that achieved by continuous heating (data not shown). These data suggest some type of antitumor activity of mild WBH alone when applied for longer durations and confirms recent findings performed on rats with mammary carcinomas (Matsuda et al., 1997). Importantly, this latter study used a technique (water bath immersion) different from the present study to achieve increased core temperatures; thus, the core temperature achieved and its duration rather than the actual technique used to apply heat seem to be important factors.

Subsequent studies revealed that, even when tumors were implanted subcutaneously (so that tumor volume changes could be more easily measured), a similar inhibitory effect of low-temperature, long-duration WBH was seen, although the magnitude of effects (in most cases) was greater when tumors were implanted abdominally. These subcutaneous studies also revealed that tumors actually exhibit a decrease in volume ($P < 0.01$ on days 2 and 4 when compared with the control). Following the initial decrease, treated tumors regrew at a rate similar to that of the control tumors. Possibilities considered regarding the mechanism of tumor volume reduction or growth delay from a single heat treatment included a direct affect of the heat treatment on tumor cell division or viability, an effect on tumor vascularization, or possibly an indirect effect on other cell types that can then kill tumor cells. Because the temperatures used in the present study were well below those known to cause thermally induced necrosis, our thoughts focused first on possible mechanisms of killing tumor cells via apoptosis, perhaps by the action of immunoeffector cells. Because SCID mice do not possess T & B lymphocytes, the only possible immune component that could be active was that composed primarily of NK lymphocytes, monocytes, and granulocytes. Because the activity of NK cells can be essentially blocked by a treatment with asialo GM1 antibody (Kasai et al., 1980; Habu et al., 1981), we examined the effects of WBH in animals given this treatment. We observed a nearly complete protection of tumor growth after WBH when animals were first pretreated with asialo GM1 antibody (Fig. 1B). There was no statistical difference in tumor volumes between the animals that were treated with both asialo GM1 antibody and hyperthermia and the control animals or control animals receiv-

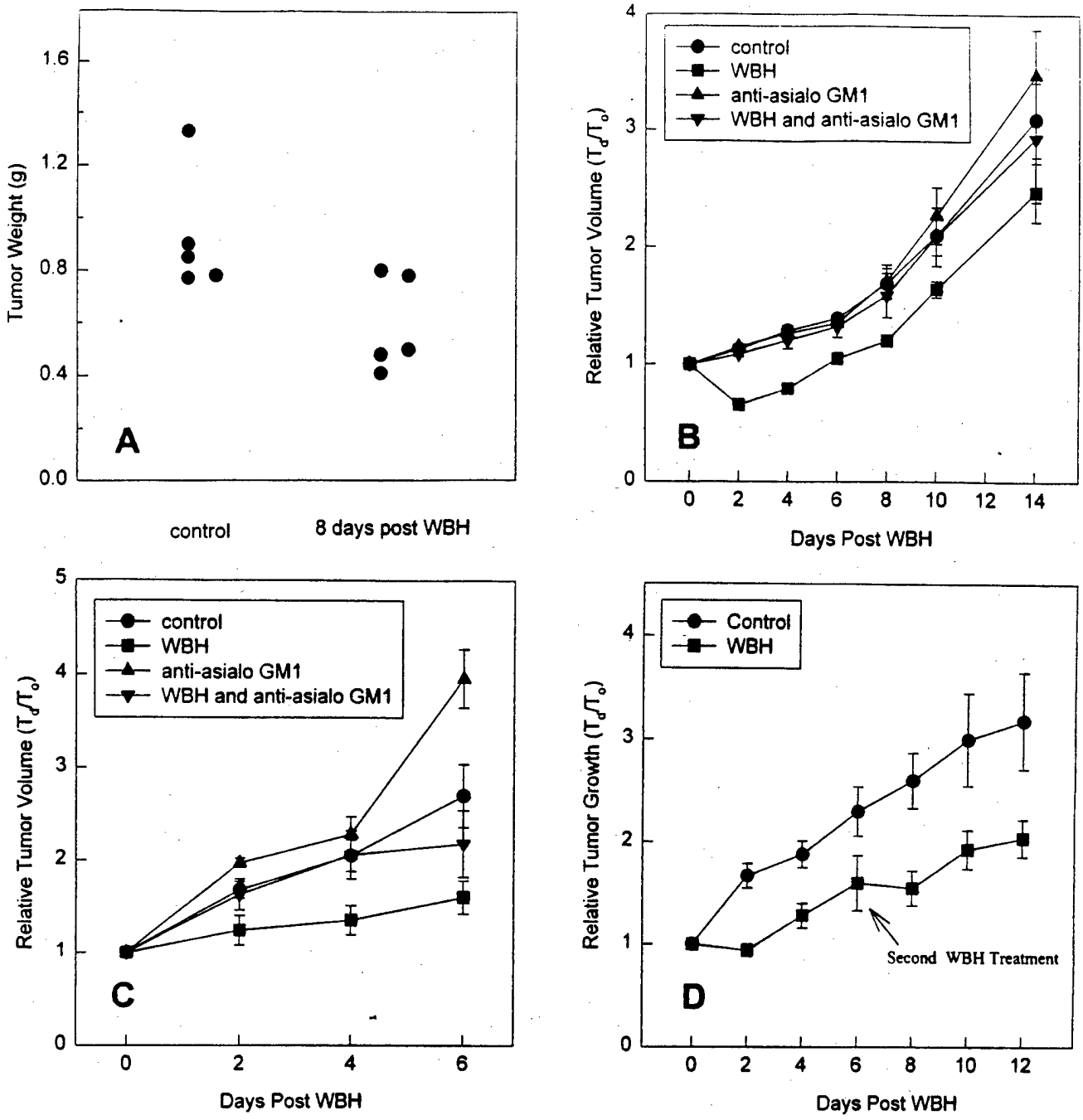



Fig. 1. **A:** Effects of 6-hr WBH on the growth of a human breast tumor implanted into the abdominal fat pad of SCID mice. Eight days after WBH, tumors were excised and weighed. **B–C:** Evidence that NK cells mediate tumor reduction following an 8-hr WBH treatment in SCID (B) and Balb/c (C) mice. SCID mice bearing a subcutaneous human breast tumor and Balb/c mice implanted subcutaneoulsy with a syngeneic tumor, colon 26, were treated with WBH with and without the depletion of NK cell activity. **D:** Effects of a second 6-hr WBH treatment on the growth of a subcutaneously implanted breast tumors in SCID mice 6 days after the initial treatment. Data are mean ± S.E. of five mice in each group.

only anti-

ing asialo GM1. These data strongly suggest that the effects of hyperthermia on tumor growth are mediated indirectly, either through hyperthermia-induced stimulation of NK cell activity or an improved access of these cells to the tumor interior.

We also performed similar WBH and NK-blocking experiments using syngeneic tumors grown in immunocompetent Balb/c mice (Fig. 1C). Although there was still a significant tumor growth delay observed in this system, we noticed that tumors that grow particularly

fast, such as many of the transplantable murine tumor cell lines, do not always show the initial decrease in volume but instead exhibit only a growth delay. Furthermore, in the more complex immunological environment of the Balb/c mouse, asialo GM1 antibody only partly blocks the effects of WBH, suggesting that other effector cells (e.g., cytolytic T lymphocytes) may help to mediate this response to WBH. As in the SCID mouse, we consistently noticed an enlarged spleen in Balb/c mice treated with long-duration mild WBH, except that the magnitude of this increase was usually greater in the Balb/c mice, especially at later time points (data not shown).

We also found that the effects of WBH treatment can be repeated, leading to larger growth delays. Figure 1D shows an example of breast tumor growth seen in SCID mice given two WBH treatments 6 days apart. Two days after the first treatment, there was a significant reduction in tumor mass ($P < 0.01$). After the second heat treatment, again there was a delay in tumor growth ($P < 0.01$).

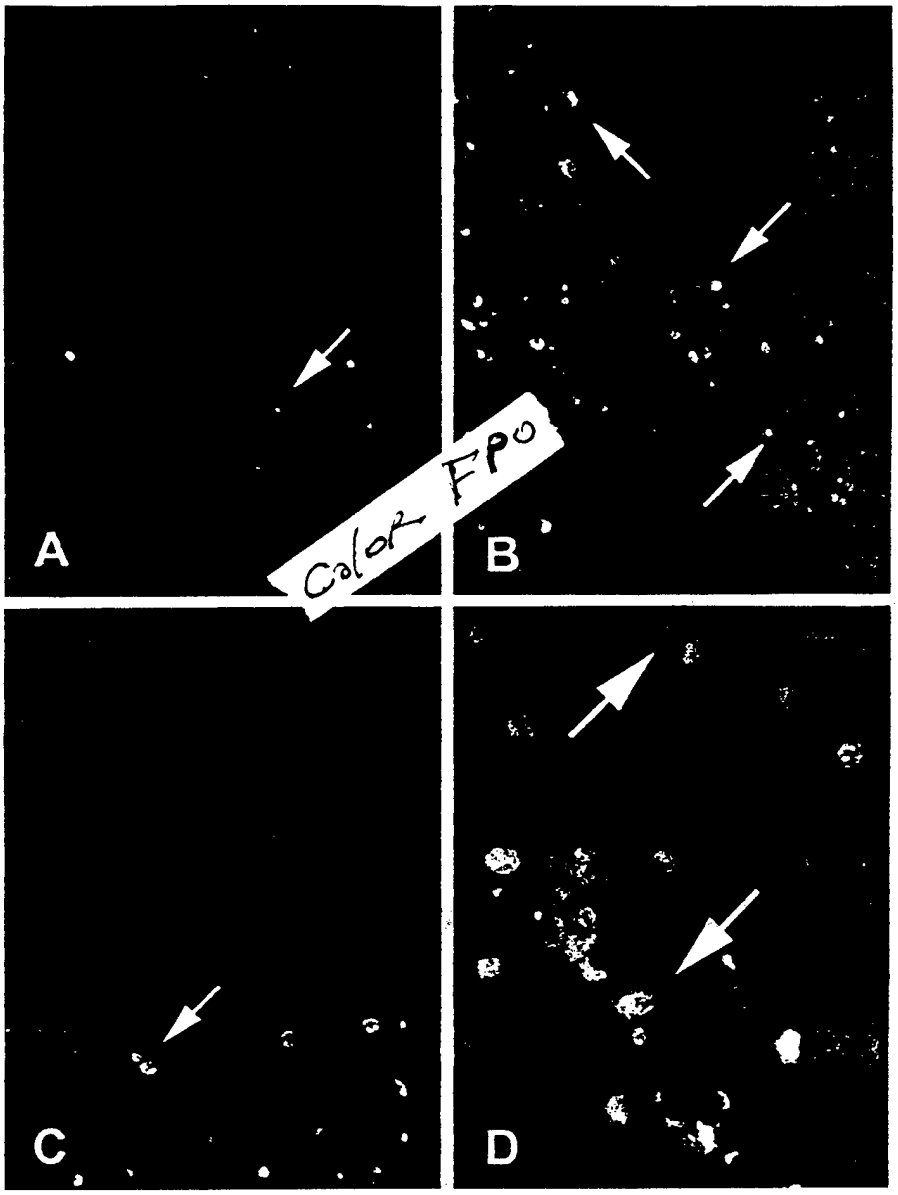
If heat-induced stimulation of NK cell activity is responsible for the antitumor effects seen in SCID mice bearing human tumors, then we should see an increase in the percentage of cells undergoing NK cell-mediated apoptosis after WBH because this is one of the known defense activities of this lymphocyte subset (reviewed by Moretta, 1997). As shown in Fig. 2, apoptosis is indeed increased in tumor cells after WBH (compare Fig. 2A and B). Importantly, if this analysis is done in animals depleted of NK cell activity prior to heat treatment, we routinely see less apoptosis in these animals than in control animals (due presumably to the elimination of the host immune response by asialo GM1 antibody), ~~despite the addition of heat, again strongly suggesting~~ that the antitumor effects are not due to a direct effect of heat alone. We observed that individual human and murine tumors differed with respect to both the ~~total percentage~~ of apoptotic cells observed and the time of the peak of apoptosis (8–16 hr). However, in every case we analyzed, there was an observable increase in apoptosis within tumors following WBH. ~~We analyzed~~ The small intestine, spleen, and thymus of Balb/c mice treated with WBH and did not find any ~~significant increase in~~ apoptosis in either of these tissues following WBH (data not shown), suggesting further that the tumor appears to be primary target of the action of the heat-stimulated activity of NK cells and that the heat treatment is nontoxic to other organs.

Figure 3A,B shows the histology of control and heated breast tumors in SCID mice 8 hr after hyperthermia treatment as viewed by hematoxylin and eosin staining. There is obvious tumor destruction, including ~~destruction~~ of small apoptotic bodies. Numerous areas containing infiltrating small mononuclear cells are evident after hyperthermia treatment; these cells appear to be composed of lymphocytelike cells (NK cells) and monocytes/macrophages and granulocytes. To identify further the infiltrating cells, frozen sections were stained with asialo GM1 antibody. Hyperthermia resulted in a substantial increase in murine NK cells that label with this antibody (Fig. 3C,D). The NK cells were present in numerous sites throughout the tumor stroma or packed into blood vessels that appeared greatly enlarged. An almost identical result was obtained when DiI-labeled human NK cells were injected into mice prior to WBH (Fig. E,F). Again, numerous human NK cells were found throughout the tumor and in the blood vessels. Very few human NK cells could be identified in the tumor in the non–heat-treated mice, ~~as illustrated in Fig. 2~~.

~~As described, long-duration, low-temperature WBH inhibits the growth of colon 26 tumors grown in the syngeneic Balb/c mouse.~~ The histological appearance of ~~these tumors~~ demonstrated areas of tumor destruction and the obvious presence of greatly enlarged blood vessels, which can still be seen 12 days after hyperthermia treatment (Fig. 4). These blood vessels often contain increased numbers of nucleated cells. Collectively, these data support the hypothesis that low-temperature, long-duration (feverlike) WBH results in a significant inhibition of tumor growth ~~resulting from~~ the action of NK cells and other effector cells of the immune response.

## DISCUSSION

Our laboratory has shown that mild WBH treatment of mice induces changes in the cytoskeletal organization and activation of protein kinase C in lymphocytes (Hughes et al., 1987; Di et al., 1997). Because of the changes observed in lymphocytes after hyperthermia and the degree of similarity this heating protocol has to the natural febrile response during infection, we have hypothesized that this mild long-duration WBH mimics the thermal element of fever and that these changes in lymphocytes may also occur in response to a natural fever. Others have recognized ~~that~~ potential similarity of WBH to fever and have hypothesized that there may be an enhancement of immune function with the use of externally applied hyperthermia (Hahn, 1982). However, although the immunological, neurological, and biochemical pathways that give rise to fever are becoming increasingly clear (Jampel et al., 1983; see reviews by Hart 1988; Moltz, 1993; Fantone and Ward 1994), there are much fewer data on the reverse, i.e., the effect of heightened body temperature on immune function in mammals, in large part because of the difficulty in separating effects that may be due to increased temperature alone from the myriad of other physiological and neurological events that occur in fever. Although it is widely assumed that there is some benefit of the elevated temperature phase of this evolutionarily conserved physiological response to infection, there is still no clear support for this assumption (Roberts, 1979; Blatteis, 1986; Duff, 1986). Many consider the benefit of higher body temperature to be on the pathogen, i.e., on reducing the replication of bacteria or viruses, whereas others have speculated that some immunological functions may be enhanced (or inhibited) by fever or externally applied hyperthermia. Further, although there have been many studies on the effects of externally applied hyperthermia on aspects of immune response (reviewed by Dickson and Shah, 1983; Tomasovic and Klostergaard, 1989), most of these studies have used much higher, unphysiological temperatures for necessarily short durations. Under these conditions, it should not be too surprising that many of these studies have actually found significant inhibition of several important immunological parameters (Izumi et al., 1983) or inhibition of the production of many cytokines




COLOR ART

Fig. 2. NK cell-mediated apoptosis is responsible for tumor volume reduction. Human breast tumors were implanted into the gonadal fat pad of SCID mice, and apoptotic cells (arrows) were visualized using the TUNEL method. **A:** Tumor from a mouse not treated with WBH. **B:** Tumor from a mouse treated with 8 hr of WBH. **C:** Tumor from a mouse in which NK cell activity was depleted before 8 hr of WBH treatment. Eight hours after WBH, tumors were removed and paraffin section were prepared for the TUNEL reaction. Sections were visualized using a fluorescence microscope. ×200. Bar = 100 μm. **D:** Magnification ×600 of B.

MOREOVER ↓ IN PARAFFIN SECTIONS

(Haveman et al., 1996). ~~Further~~, whether externally applied hyperthermia can actually mimic the thermal element of the much more physiologically complex fever is not known. Nevertheless, the recent important demonstration (Matsuda et al., 1997) that WBH administered in a feverlike fashion (i.e., at much lower temperatures and at longer durations than have been conventionally used) results in an inhibition of tumor growth makes further investigation of this protocol and its mechanism(s) of action of great importance for future clinical application.

In the present study, we found a significant enhancement of the activity of at least one set of lymphocytes, (THE NK cells), in response to low-temperature, long-duration WBH and provided evidence for enhanced recruitment and activity of other effector cells. The activity of ~~effector~~ NK cells was linked to a significant increase in apoptosis, which resulted in a reduction of tumor volume or a growth delay. Others have previously shown that NK cells cultured in vitro after isolation from normal volunteers and cancer patients who underwent short-duration WBH had increased activity when tested in chromium release assays using NK cell-sensitive targets (Zanker and Lange, 1982; Lackovic et al.,

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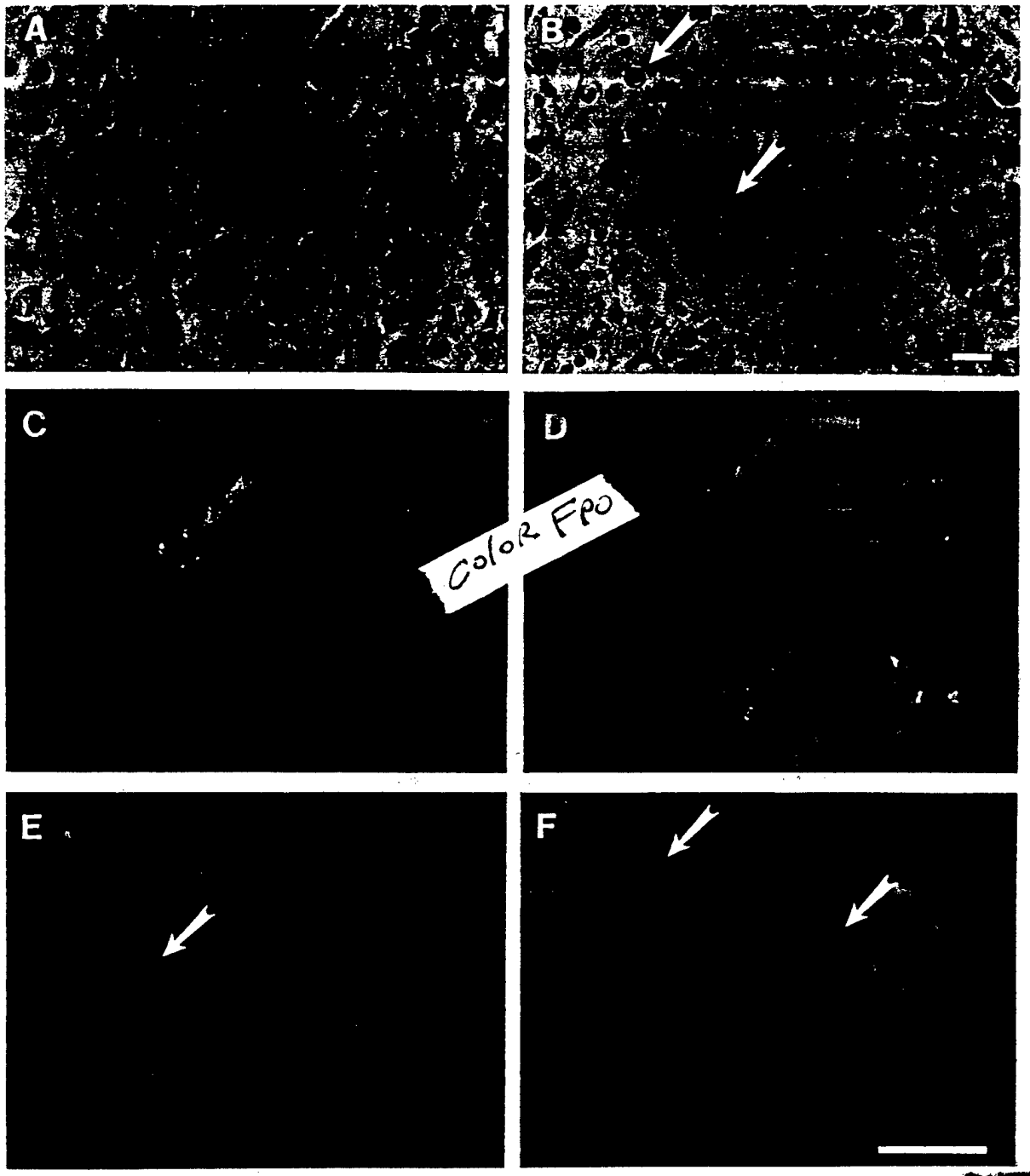


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Fig. 3. Recruitment of immune cells to human breast implanted in the gonadal fat pad of SCID mice after WBH. Histological section of a tumor from a mouse not treated with WBH (**A**) and of a tumor from an mouse treated with 8 hr of WBH (**B**), showing tumor destruction and the presence of an infiltrate (arrow) composed of NK cells, monocytes/macrophage, and granulocytes. Tumors were removed 8 hr after WBH, and paraffin sections were stained with hematoxylin and eosin. ×600. Bar, 30 μm. Fluorescent staining of murine NK cells (arrows) in a tumor from a mouse not treated with WBH (**C**) and in a tumor from a mouse treated with 8 hr of WBH (**D**). Tumors were removed 8 hr after WBH, and cryostat sections were stained with asialo GM1 FITC. Sections were then viewed using a confocal microscope. ×600. Fluorescent identification DiI-labeled human NK cells (arrows) in a tumor from a mouse not treated with WBH (**E**) and in a tumor from a mouse treated with WBH for 8 hr (**F**). DiI-labeled human NK cells were injected 24 hr before WBH. Immediately after treatment, the tissue was place in Tissue Tek O.C.T. compound and frozen. Cryostat sections were observed with a fluorescent microscope. ×1,000. Bar, 30 μm.

TR UP

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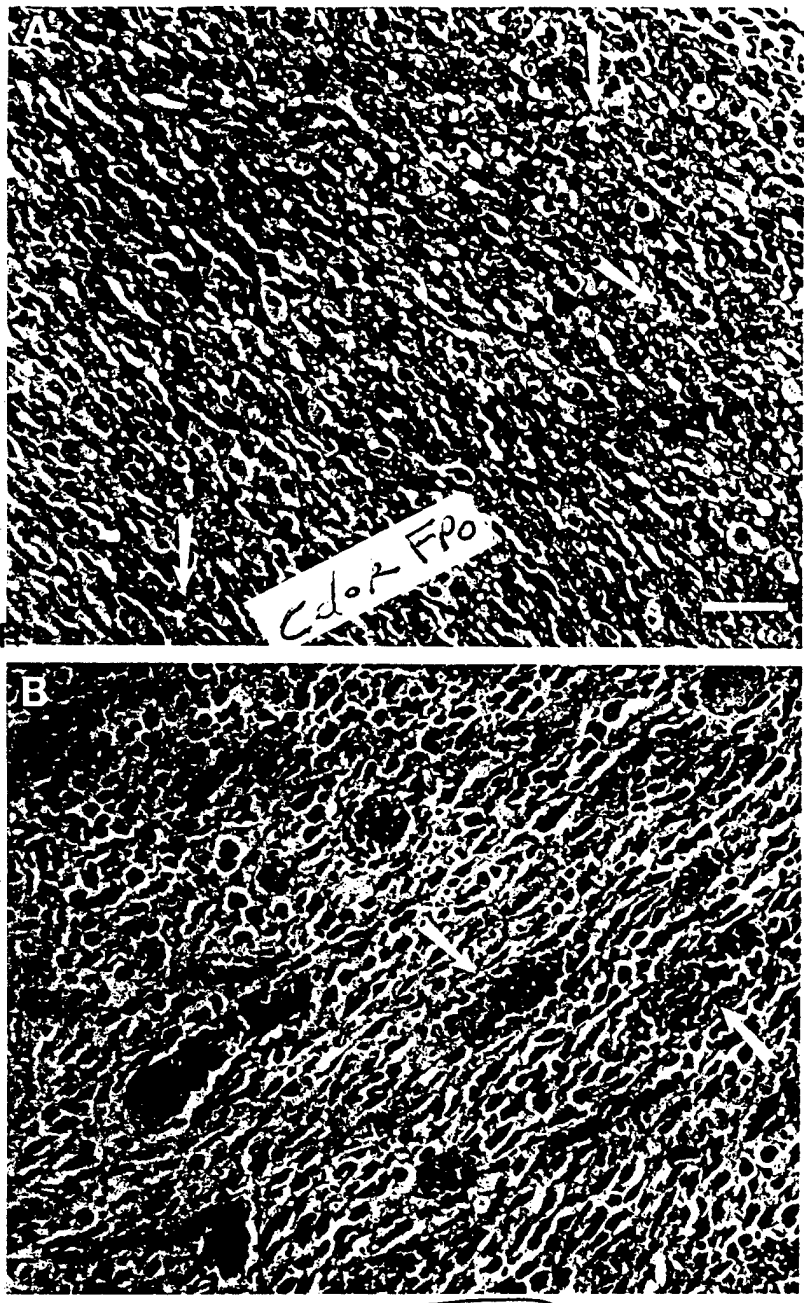


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Fig. 4. Enlargement of tumor blood vessels after 8 hr of WBH. Colon 26 tumors from a (A) mouse not treated shows normal blood vessels (arrows) and from a (B) mouse treated with 8 hr of WBH shows enlarged blood vessels (arrows). Two weeks after WBH, tumors were removed, and paraffin sections were stained with hemotoxylin and eosin. ×200. Bar, 100 μm.

1988; Park et al., 1990), whereas other studies have shown that NK cell activity decreases when cells are heated at these same mild temperatures in vitro (Dinarello et al., 1986; Azocar et al., 1992). However, the data that are presented in the present study show for the first time that in vivo antitumor activity of host and adoptively transferred NK cells is increased following mild WBH and that these and other cells migrate to the site of tumor in much greater numbers following WBH. In SCID mice, where the role of NK cells can be most clearly studied (because other, more specific, immune effector cells are absent), a single treatment of WBH caused a rapid decrease in tumor volume that persisted for a short period of time. The duration of heating needed to obtain a significant tumor reduction and/or delay was determined in preliminary studies to be at least 6 hr. There is little difference in the response of an individual tumor to a 6-hr or 8-hr treatment. However, the growth rate and hyperthermia response differ between different patient tumors (compare Fig. 1A with B). The effect of hyperthermia can be repeated with the addition of a second heat treatment, leading to greater volume differences, but further study on fractionation of heat treatments could result in a more sustained and complete antitumor effect.

The antitumor effect was almost entirely blocked by the addition of asialo GM1 antibody. Asialo GM1 antibody has little cross reactivity with other immune sub-

sets and can essentially block NK cell activity as the dose that it was administered. This provides evidence that NK cells were the predominate factor mediating the antitumor effect induced by WBH.

WBH had a similar effect on tumor growth in Balb/c mice except that, when these immunocompetent mice were treated with WBH, there was a tumor growth delay that persisted for a longer period of time; this result suggests that, in the presence of a more complete immune system, the heat-induced stimulation of immune effector cells may result in a more sustained effect. In this model, the addition of asialo GM1 antibody only partly blocked the effects of WBH. These results indicate that other effector cells, such as cytotoxic T lymphocytes, may have a role.

The antitumor effects of low-temperature, long-duration WBH may be mediated through a direct effect of heat on immune effector cells or through other pathways that affect the activity of NK and other lymphocyte subsets. One likely system may be the induction of cytokines, for example, the interferons (IFN). The production of IFN-γ has been shown to increase in PHA-stimulated cultures isolated from monkeys (Downing et al., 1985) and humans (Park et al., 1990) treated with mild WBH. Other likely possibilities may involve cytokine-mediated changes in adhesive and/or migration potential of lymphocytes. Recent studies have already suggested that structural and functional changes occur in lymphocytes in response to low-temperature, long-duration WBH, which would very likely affect their motility or migration (Hughes et al., 1987; Di et al., 1997). Further, it has recently been shown that the function of an adhesion molecule on the surface of lymphocytes (L-selectin) is increased following an in vitro low-temperature, long-duration hyperthermia treatment (Wang et al., 1998).

In our experiments, we found a greater number of NK cells in the tumor vasculature and in the tumor tissue immediately after WBH. Few NK cells were present in nontreated tumors, but a basal level of primary tumor control by NK-mediated apoptosis does exist. We found that we could consistently suppress this basal level by administering continual injections of asialo GM1 antibody and promote the growth of human tumors in our SCID model. Furthermore, tumor-bearing mice injected with asialo GM1 antibody (SCID and Balb/c) had shorter survival times (data not shown), and control animals treated with asialo GM1 antibody grew at a faster rate, indicating that a basal level of host NK cell mediated cytotoxicity somewhat inhibits tumor growth in rats, asialo GM1 treatments can enhance tumor incidence and growth rate of 3-methyl cholanthrene-induced tumors (Mather et al., 1994).

NK cells are surveillance lymphocytes that can potentially kill circulating tumor cells and metastatic foci (Gorelik et al., 1982). The presence of a large number of NK cells in a primary tumor is uncommon, but they have been seen infiltrating metastatic melanoma in humans (Kornstein et al., 1987). Also, adoptively transferred NK cells have been shown to be in direct contact with metastatic tissue and induce apoptosis of tumor cells (Okada et al., 1996). The increase in tumor cell apoptosis observed in the present study could be explained by a greater number in NK cells at the site of tumor and/or by an increase in specific NK cell activity. It is possible that WBH increases the ability of NK cells to adhere to blood vessels and migrate into tumor tissue. NK cell accumulation could also be caused by an increase in blood flow through the tumor after WBH. The enlarged appearance of blood vessels shown in the present study support this possibility. There is a long-standing recognition that the intratumor pressures may serve effectively to block the entry of various types of therapies or immunoeffector cells (Jain, 1997). The application of WBH may allow for a sustained vasodilatation of the vessels within the tumor, thereby reducing the intratumor pressure and allowing a greater delivery of immunoeffector cells into the tumor. As shown in the present study, enlarged blood vessels are present in the colon 26 tumor up to the time of resection (1–2 weeks after hyperthermia). This long-term effect on the vasculature in the tumor also are suggestive of the development of conditions that are similar to those that occur in an inflammatory response.

The possibility that WBH causes changes in tumor cells that affect NK cell activity and/or recruitment, such as the expression of new molecules on the surface of the tumor, has not been addressed here. A study by Fujieda et al. (1995) has shown that heat shock enhances the susceptibility of tumor cells to lysis by lymphokine-activated killer cells. Other studies by Multhoff et al. (1995a,b) have suggested that the expression of a heat shock protein on the surface of tumor cells may serve as receptors for NK cell killing. In support of this possibility, we found that the expression of several heat shock proteins are significantly increased on human breast tumors after WBH (X.-Y. Wang, R. Burd, E. Repasky, and J. Subjeck, manuscript in preparation). Other possibilities include alterations in MHC I, which acts as killer inhibitory receptors (Moretta et al., 1990).

Although many questions are raised by these data, these somewhat surprising effects of low-temperature (fever range) WBH strongly suggest that there is an antitumor immune effect, which if better understood, may be improved and exploited for therapeutic use. Particularly appealing is the possibility that this treatment may work well as a new adjuvant and may be applied with other therapies designed to stimulate the existent immune response, i.e., cytokine treatments, adoptive therapies, or gene-based therapies, all of which help to target the tumor by immune system-mediated cell killing. However, if we are simply mimicking the thermal element of a fever, perhaps this treatment is not "new" at all but in fact has been conserved through millions of years of evolution because of the potential benefit of transient hyperthermia to the immune response. Thus, it is important to define as soon as possible the various immunological parameters that may be affected by this treatment. For example, what is the role of cytokines in the observed antitumor effects? Are there certain cytokines that are actually induced by the low-temperature, long-duration WBH that may help to stimulate the antitumor immune response? Studies by d'Oleire (1993) and Robins et al. (1995), using high-temperature protocols, have indicated that some cytokines such as interleukin (IL)-1β, IL-6, IL-10, GM-CSF, and TNF-α are induced following WBH, whereas other studies have indicated

that the production of many cytokines is actually inhibited (Haveman et al., 1996). A similar study using low-temperature, long-duration WBH has not yet been conducted. Along these same lines, it will be important to determine if exogenous addition of cytokines could be synergistic with mild WBH in the treatment of tumors. Further studies on the potential mechanism(s) of increased antitumor effects caused by low-temperature, long-duration (fever range) WBH may lead to the development of this procedure as a useful and safe addition to cancer therapy.

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# Tumor cell apoptosis, lymphocyte recruitment and tumor vascular changes are induced by low temperature, long duration whole body hyperthermia

RANDY BURD, THOMAS S. DZIEDZIC, YAN XU, MICHAEL A. CALIGIURI*,

JOHN R. SUBJECK** & ELIZABETH A. REPASKY

*Departments of Molecular Immunology, Medicine** and

*Molecular and Cellular Biology***, *Roswell Park Cancer Institute*

*Buffalo, New York 14263*

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Address correspondence to:

Elizabeth A. Repasky, Ph.D.

Dept. of Molecular Immunology

Roswell Park Cancer Institute

Buffalo, New York 14263

Phone: 716-845-3133

Fax: 716-845-8906

e-mail: repasky@sc3101.med.buffalo.edu

Running Head: Mild hyperthermia and tumor growth

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**Abstract:**

A single treatment of low temperature, long duration whole body hyperthermia of mice bearing either human breast tumors or transplantable, syngeneic murine tumors can cause a temporary reduction of tumor volume and/or a growth delay. In both systems, this inhibition is correlated with the appearance of large numbers of apoptotic tumor cells. Since this type of mild heat exposure, comparable to a common fever, is not itself directly cytotoxic, other explanations for the observed tumor cell death were considered. Our data support the hypothesis this hyperthermia protocol stimulates some component(s) of the immune response which results in increased anti-tumor activity. In support of this hypothesis, increased numbers of lymphocyte-like cells, macrophages and granulocytes are observed in the tumor vasculature and in the tumor stroma immediately following this mild hyperthermia exposure. In Balb/c mice, an infiltrate persists for at least two weeks. Using the SCID mouse/human tumor system, we found that both host NK cells, and injected human NK cells were increased at the site of tumor following hyperthermia treatment. Experiments using asialo-GM1 antibodies indicate that the tumor cell apoptosis seen in the SCID mouse appears to be due almost entirely to the activity of at least NK cells, although additional roles for other immunoeffector cells and cytokines appear likely in the immunologically complete Balb/c model. Another interrelated hypothesis is that immunoeffector cells may have greater access to the interior of the tumor since we have observed that this treatment causes an obvious expansion in the diameter of blood vessels within the tumor and an increase in nucleated blood cells within the vessels, which persists as long as two weeks following treatment. Further study of the mechanisms by which mild hyperthermia exerts anti-tumor activity could result in this treatment protocol being used as an effective, non-toxic adjuvant to immunotherapy and/or other cancer therapies.

## Introduction

There are compelling biological reasons which continue to drive a century-long interest in the use of hyperthermia in the treatment of human cancer (Hahn,1982; Oleson *et al.*, 1988). The origins of hyperthermia as a cancer treatment modality are linked with some of the earliest immunotherapy literature since it has been recognized that the bacterial toxins given to cancer patients to stimulate their immune system also gave rise to high fevers; subsequently it was observed that those who developed the highest fevers were most often the ones who had the longest survival (Nauts, 1990). In early literature, it was also noted that individuals who survived infectious disease associated with fever may exhibit tumor regression (e.g., Westermark, 1927; Busch, 1866). However, modern clinical hyperthermia efforts to treat human cancers do not utilize fever as the source of heat, but instead have focused primarily on the use of externally applied heat (usually by using microwaves) directed at only a limited region of the body for a short duration. Although the physics behind delivery of effective 'local hyperthermia' to tumors is complex, many years of experimental study (Oleson *et al.*, 1988) led to the initiation of several clinical trials in the mid-eighties which met with contradictory results; however, several more recent prospective randomized trials have now plainly demonstrated the significant potential of local hyperthermia to improve antitumor effects when combined with either chemotherapy or radiation for several different types of human cancers (Seachrist, 1993; Valdagni and Amichetti, 1993; Sugimachi *et al.*, 1995; Overgaard *et al.*, 1996; Vernon *et al.*, 1996).

Although local hyperthermia was initially envisioned as a type of thermal radiation therapy where a heat dose hot enough to kill cancer cells directly would be selectively delivered to the tumor mass, it has been noted in the last few years that the tumor temperatures actually achieved during treatment *are often well below those known to be required for any direct cytotoxic effect*

(Oleson, 1995). It has been proposed therefore that the clinical benefit of local hyperthermia is more likely the result of the low heat temperatures achieved, indirectly improving radiation response by increasing tumor oxygenation via increasing blood flow into the tumor. Indeed, studies from several other groups have focused specifically on radiobiological effects of 'low temperature' local hyperthermia, in particular the effects of low (heating) temperature on radiosensitization and reoxygenation, and alterations in blood flow (e.g. Armour *et al.*, 1991; Hetzel *et al.*, 1992; Armour *et al.*, 1993; Horsman and Overgaard, 1996; Song *et al.*, 1996a; Song *et al.*, 1996b).

While local and often regional hyperthermia have been the primary means of applying high temperature hyperthermia, studies over many years have also investigated the effectiveness of whole body heating (e.g. Robins *et al.*, 1993). In this procedure, the core temperature of the animal or patient is usually raised to a very high temperature range(approximately 41.8 - 42°C) which can be sustained for a only a short time (30 min to 2 hr) (Pettigrew *et al.*, 1974; Bull, 1983). The conventional (high temperature, short duration) WBH protocol in combination with various chemotherapeutic drugs results in enhanced cytotoxicity of both tumor and normal tissue, which seriously reduces therapeutic gain (Gerad *et al.*, 1983; Wondergem *et al.*,, 1988; Ohno *et al.*, 1991). However a novel low temperature, (i.e., mild hyperthermia) protocol, *which can be applied for much longer periods of time*, was found to significantly improve anti-tumor effects without added normal tissue toxicity from carboplatin and tumor necrosis factor (Sakaguchi *et al.*, 1994). Surprisingly, when the low temperature, long duration WBH procedure was applied alone, using a rat tumor model, *without any other additional therapy*, a tumor growth delay was still observed, together with a significantly (50%) reduced incidence of lymph node metastases (Matsuda *et al.*, 1997).

One of the most important biological questions arising from these recent studies concerns the mechanism by which such a mild systemic hyperthermia treatment applied for a long duration produces such significant albeit temporary anti-tumor responses. Recent studies from our laboratory indicate that a nearly identical, low temperature, long duration whole body heat exposure in mice leads to several important structural and functional changes in lymphocytes, suggesting that this treatment may be able to stimulate the activity of various immune effector cells (Di *et al.*, 1997). Here we present several studies on the effects of low temperature, long duration whole body hyperthermia on the growth and histological appearance of human and murine tumors. Our studies reveal that a mild heating of animals bearing tumors for a period of several hours results in a significant lymphoid infiltrate and tumor cell apoptosis due to (at least) the activity of natural killer lymphocytes. These effects are seen with a simultaneous expansion of the volume of tumor vasculature which could be useful for enhancing the targeting of immunoeffector cells, and the delivery of reagents into the tumor interior. While the mechanism(s) of these responses to elevated body temperature induced by external means is not yet known, it is highly likely that we are exploiting a heat-sensitive, physiological response already in place that helps stimulate immunoeffector activity during a fever.

## Materials and Methods

**Tumor implantation/growth.** For some experiments, specimens of human breast tumors were embedded into the abdominal gonadal fat pad of SCID mice (Sakakibara *et al.*, 1996) and allowed to grow for 3 weeks before receiving hyperthermia treatment. For other experiments, human breast tumors that had been passaged several times in SCID mice were dissected into small pieces (1-2 mm in diameter) and implanted subcutaneously into SCID mice. Murine colon 26 tumors were injected subcutaneously into Balb/c mice from a single cell suspension ($1 \times 10^6$ cells/ml). Hyperthermia was initiated after the tumors reached a volume of $\sim 40 mm^3$. The tumors were measured using a venier caliper every 2 days to determine the shortest diameter (A) and the longest diameter (B). The volume was then calculated using the formula $V=(A^2B)/2$. The relative tumor growth ($T_r$) was then calculated for each tumor by dividing the volume of the tumor at any given day after WBH ($T_d$) by the volume at the start of WBH ($T_o$).

**Whole body hyperthermia treatment.** Mice were placed in microisolator cages preheated to 38°C that contain food, bedding and water. The cages are then placed in a gravity convection oven with preheated incoming fresh air (Memmert model BE500, East Troy, WI). The average body temperature of the mice before WBH is 37.5C. The temperature was then increased gradually (1°C) every 30 min until a core temperature of 39.8°C (+/-0.2°C) was achieved. The temperature was then maintained for 8h. During the heating, the core temperatures of the mice were monitored continuously by an inserted rectal thermal couple (prepared from fine wire) taped to the tail (Doric, Tredicator 412A, accuracy of +/- 0.1°C). The thermocouple and readout were calibrated against a National Bureau of Standards calibrated mercury thermometer before each experiment. The body temperature was also monitored with the Electronic Laboratory Animal Monitoring System from Biomedic Data Systems (Maywood, NJ). Microchip transponders 14 mm

x 2.2 mm were subcutaneously implanted into the dorsal thoracic area using a trocar. Temperatures were collected using a scanner/readout. We have found that it was not difficult to maintain core temperatures between 39°C and 40°C. When heating several animals, internal variability between individual animals was +/- 0.2°C.

**Depletion of murine immune cells.** To deplete NK cell activity, asialo GM-1 antibody (Wako Chemicals, Richmond, VA) was diluted 1:5 in PBS and 100 $\mu$l was injected i.p. 3 days prior to WBH and every 5 days thereafter.

**Immunofluorescent staining.** Tumors were removed from the gonadal fat pad, immediately immersed in Tissue Tek O.C.T. compound (Miles, Elkhart, Ind.), and frozen in liuqid nitrogen. Sections (10 $\mu$m) were cut on a cryostat, placed on slides and allowed to dry for 30 min at room temperature (RT). Slides were stored at -70°C until used. For immunofluorescent staining with asialo GM-1 antibody, a two layer technique was used (Parr *et al.,* 1990). Slides were first air dried for 30 min RT, or fixed 10 min in acetone. The slides were then incubated with 10% normal goat serum/PBS to block non-specific binding. Rabbit anti-asialo GM-1 antibody (1:250) was applied for 2hr as the first layer. As a control, duplicate sections were incubated with 10% normal rabbit serum for 2hr. A FITC conjugated goat anti-rabbit antibody (1:200) was applied for 1h as the second layer. Propidium iodide was used as a counterstain. Slides were viewed using a confocal microscope.

**Labeling human NK cells.** Highly purified human NK cells ($5x10^6$ cells/ml) ,were incubated with 2 uM DiI for 30 min on ice. The cells were washed 3 times in PBS and then $2x10^6$ cells were injected i.p. into mice bearing abdominally implanted human breast tumors 24 hr. before hyperthermia. Immediately following treatment the tumors were removed and frozen sections were prepared as described.

**Detection of apoptosis.** Apoptosis was detected using the Oncor ApopTag Plus *In Situ* Apoptosis Detection Kit, Gaithersburg, MD. Briefly, tumors were removed 8-16 hrs following WBH and fixed in 4% formalin for 14 hr. Paraffin sections were treated with proteinase K for 20 min. TdT enzyme was then added for 1 hr at 37°C to label fragmented DNA ends with digoxigenin-nucleotides. Anti-digoxigenin fluorescence antibody was then added for 30 min. A wet mount was prepared using propidium iodide and the slides were observed under a fluorescent microscope.

## Results

Two tumor models were analyzed in this study, 1) SCID mice implanted with human primary breast tumors (Sakakibara *et al.*, 1996), and 2) Balb/c mice implanted with syngeneic tumors. As shown in Figure 1 (a), when mice bearing abdominally implanted breast tumors are given low temperature, long duration WBH using a warm air environment technique and the tumors are excised and weighed 8 days later, they are found to be significantly lower in weight. The control group had an average weight of 1g (+/- 0.1g), while the treated group had an average weight of 0.6g (+/-.08g , $p < .05$). The mice themselves were not seen to differ in any other way; other internal organs appeared completely normal, except that heated mice are often seen to have an enlarged spleen. Other experiments revealed little or no effect on tumor volume if the hyperthermia treatment was given for 2 or 4 hr. These data suggest some type of anti-tumor activity of mild WBH alone, applied for longer durations, and confirms recent findings performed on rats bearing mammary carcinomas (Matsuda *et al.*, 1997). Importantly, this latter study used a different technique (water bath immersion) from the present study to achieve increased core temperatures; thus it appears as though the core temperature achieved (and its duration) are important factors, rather than the actual technique used to apply heat.

Subsequent studies revealed that even when tumors were implanted subcutaneously, (so that tumor volume changes could be more easily measured) a similar inhibitory effect of low temperature, long duration WBH was seen, although in most cases, the magnitude of tumor reduction was greater if tumors were implanted abdominally. These subcutaneous studies also revealed that tumors actually exhibit an initial decrease in volume (seen within 48 hr following WBH) which is followed by a growth delay (Fig.1b). Possibilities regarding the mechanism of tumor volume reduction or growth delay from a single heat treatment included a direct affect of

the heat treatment on tumor cell division or viability, an effect on tumor vascularization or possibly an indirect effect on other cell types which can then kill tumor cells. Since the temperatures used in this study are well below those known to cause thermally induced necrosis, our thoughts focused first on possible mechanisms of killing of tumor cells via apoptosis, perhaps by the action of immunoeffector cells. Since SCID mice do not possess T or B lymphocytes, the only possible immune component which could be active was that composed primarily of natural killer (NK) lymphocytes, monocytes and granulocytes. Since the activity of NK cells can be essentially blocked by a treatment with asialo GM-1 antibody (Kasai *et al.*, 1980), we examined the effects of WBH in animals given this treatment. As can be seen in Figure 1b, we observed a nearly complete protection of tumor growth following WBH if animals were first pre-treated with asialo GM-1 antibody. Control animals with asialo GM-1 antibody grew at a faster rate indicating that a basal level of host NK cell-mediated cytotoxicity somewhat inhibits tumor growth. These data strongly suggest that the effects of hyperthermia on tumor growth are mediated indirectly, either through hyperthermia-induced stimulation of NK cell activity, and/or an improved access of these cells to the tumor interior.

We also performed similar WBH and NK-blocking experiments using syngeneic tumors grown in immunocompetent Balb/c mice (Fig. 1c). While there is still a significant tumor growth delay observed in this system, we have noticed that tumors which grow particularly fast such as many of the transplantable murine tumor cell lines, do not always show the initial decrease in volume, but instead exhibit only a growth delay. Furthermore, in the more complex immunological environment of the Balb/c mouse, asialo GM-1 antibody only partially blocks the effects of WBH suggesting that other effector cells (e.g., cytolytic T lymphocytes) may help to mediate this response to WBH. As in the SCID mouse, we consistently noticed an enlarged spleen

in Balb/c mice treated with long duration, mild hyperthermia, except that usually the magnitude of this increase was usually greater in the Balb/c mice especially at later time points (data not shown).

We have also found that the effects of WBH treatment can be repeated, leading to larger growth delays. Shown in Figure 1d is an example of breast tumor growth seen in SCID mice given two WBH treatments 6 days apart; following the second heat treatment, there is again a delay in tumor growth.

If a heat induced stimulation of natural killer cell activity is responsible for the anti-tumor effects seen in SCID mice bearing human tumors, then we should see an increase in the percentage of cells undergoing NK cell-mediated apoptosis post WBH since this is one of the primary defense activities of this lymphocyte subset (reviewed by Moretta, 1997). As shown in Fig. 2, apoptosis is indeed increased in tumor cells following WBH (compare Figs. 2a and 2b). Importantly, if this analysis is done in animals depleted of NK cell activity prior to heat treatment, we routinely see *less* apoptosis in these animals than in control animals (due presumably to the elimination of the host immune response by asialo GM-1 antibody) despite the addition of heat, again strongly suggesting that the anti-tumor effects are not due to a direct effect of heat alone. We have observed that individual human and murine tumors differed with respect to both the total percentage of apoptotic cells observed, as well as the time of the peak of apoptosis (ranging between 8 -16 hr); however, in every case we have analyzed, there is an observable increase in apoptosis within tumors following whole body hyperthermia. We have analyzed the small intestine and the thymus of mice treated with WBH and do not find any significant increase in apoptosis in either of these tissues following WBH (data not shown), suggesting that the tumor is the primary target of the action of the heat-stimulated activity of NK cells.

Figure 3a,b shows the histology of control and heated breast tumors in SCID mice 8 hrs post hyperthermia treatment as viewed by hematoxylin and eosin staining. Numerous areas containing infiltrating small mononuclear cells are evident after hyperthermia treatment; these cells appear to be composed of lymphocyte-like cells (NK cells) and monocytes/macrophages and granulocytes. To further identify the infiltrating cells, frozen sections were stained with asialo GM1 antibody. As shown in Figs. 3c and d, hyperthermia results in a substantial increase in murine NK cells. The NK cells were present in numerous sites throughout the tumor stroma, or packed into blood vessels that appear greatly enlarged. As shown in Figs. 3e and f, an almost identical result was obtained when DiI labeled human NK cells were injected into mice prior to WBH. Again numerous human NK cells were found throughout the tumor and in the blood vessels. Very few human NK cells could be identified in the tumor in the non-heat treated mice.

As shown above, long duration, low temperature WBH also inhibits the growth of colon 26 tumors grown in the syngeneic Balb/c mouse, and causes a significant increase in the number of cells undergoing apoptosis, and infiltrating immune cells. The histological appearance of these tumors demonstrates areas of tumor destruction, and the obvious presence of greatly enlarged blood vessels, which can still be seen 12 days after the hyperthermia treatment (Fig. 4). Often these blood vessels contain increased numbers of nucleated cells. Collectively, these data support the hypothesis that low temperature, long duration (fever-like) WBH results in a significant, inhibition of tumor growth resulting from the action of NK cells and other effector cells of the immune response.

## Discussion

Our laboratory has previously shown that mild, long duration WBH treatment of mice induces changes in the cytoskeletal organization and activation of protein kinase C in lymphocytes (Di et al, 1997). Because of the changes observed in lymphocytes following hyperthermia and the degree of similarity this heating protocol has to the natural febrile response during infection, we have hypothesized that this mild, long duration WBH mimics the thermal element of fever and that these changes in lymphocytes may also occur in response to a natural fever. In the past others have recognized that potential similarity of WBH to fever, and have hypothesized that there may be an enhancement of immune function with the use of externally applied hyperthermia (Hahn, 1982). However, while the immunogical, neurological and biochemical pathways which give rise to fever are becoming increasingly clear, (Jampel *et al.*, 1983; see reviews by Fantone and Ward 1994; Hart 1988; Moltz, 1993) there is in fact much less data on the reverse, i.e., the effect of heightened body temperature on immune function in mammals, in large part because of the difficulty in to separating effects that may be due to increased temperature alone from the myriad of other physiological and neurological events which result in fever. While it is widely assumed that there is some benefit of the elevated temperature phase of this evolutionarily conserved physiological response to infection, there is still no clear support for this assumption (Roberts, 1979; Blatteis, 1986, Duff, 1986). Many consider the benefit of higher body temperature to be on the pathogen, i.e., on reducing the replication of bacteria or viruses, while others have speculated that some immunological functions may be enhanced (or inhibited) by fever or externally applied hyperthermia. Further, while there have been many studies on the effects of externally applied hyperthermia on aspects of immune response (reviewed by Tomasovic and Klostergaard, 1989), most of these studies have used much higher, unphysiological temperatures, for necessarily short durations. Under these conditions, it

should not be too surprising that many of these studies have actually found significant inhibition of several important immunological parameters (Izumi *et al.*, 1983). Further, whether externally applied hyperthermia can actually mimic the thermal element of the much more physiologically complex fever is not known. Nevertheless, the recent important demonstration (Matsuda *et al.*, 1997) that WBH administered in a fever-like fashion (i.e., at much lower temperatures, and at longer durations than have been conventionally used in the past) results in an inhibition of tumor growth, makes further investigation of this protocol and its mechanism(s) of action of great importance for future clinical application.

In this study, we have found a significant enhancement of the activity of at least one set of lymphocytes, NK cells, in response to low temperature, long duration WBH and provide evidence for enhanced recruitment and activity of other effector cells as well. The activity of at least NK cells was linked to a significant increase in apoptosis, which resulted in a reduction of tumor volume or a growth delay. Others have previously shown that NK cells cultured *in vitro* after isolation from normal volunteers and cancer patients who underwent short duration, WBH had increased activity when tested in chromium release assays using NK cell sensitive targets (Lackovic *et al.*, 1988; Park *et al.*, 1990; Zanker and Lang, 1982), while other studies show that NK cell activity decreases when cells are heated at these same mild temperatures *in vitro* (Azocarl *et al.*, 1992; Dinarello *et al.*, 1986). However, the data that is presented in the present study show for the first time that *in vivo* anti-tumor activity of host and adoptively transferred NK cells is increased following mild WBH, and that these cells migrate to the site of tumor in much greater numbers following WBH. In SCID mice where the role of NK cells can be most clearly studied (since other, more specific immune effector cells are absent) a single treatment of WBH was found to cause a rapid decrease in tumor volume that persisted for a short period of time; however, the effect could be repeated upon the addition of

a second heat treatment. Further study on fractionation of heat treatments may result in a more sustained and complete anti-tumor effect. The anti-tumor effect was almost entirely blocked by the addition of asialo GM-1 antibody, providing evidence that NK cells were the predominate factor mediating the anti-tumor effect induced by WBH.

WBH had a similar effect on tumor growth in Balb/c mice. When Balb/c mice were treated with WBH, there was a tumor growth delay that persisted for a longer period of time; this suggests that in the presence of a more complete immune system, the heat-induced stimulation of immune effector cells may result in a more sustained effect. In this model, the addition of asialo GM-1 antibody only partially blocked the effects of WBH. These results indicate that other effector cells, such as cytotoxic T lymphocytes, may have a role.

The anti-tumor effects of low temperature, long duration WBH may be mediated via a direct effect of heat upon immune effector cells, or through other pathways which indirectly affect the activity of NK and other lymphocyte subsets. One likely system may be the induction of cytokines, such as the interferons (IFN) through heat treatment. The production of IFN-$\gamma$ has been shown to increase in PHA stimulated cultures isolated from monkeys (Downing *et al.*, 1985) and individuals (Park *et al.*, 1990) treated with mild WBH. Furthermore, the anti-tumor activity of NK cells can be enhanced in mice by using IFN inducers such as poly I:C (Gresser *et al.*, 1978) or LCMV infection (Welch and Zinkerangel, 1977). Other likely possibilities may involve cytokine mediated changes in adhesive and/or migration potential of lymphocytes. Recent studies from our group already suggest that structural and functional changes occur in lymphocytes in response to low temperature, long duration WBH which would very likely affect their motility or migration (Hughes *et al.*, 1987; Di *et al.*, 1997). Further, we have recently shown that the function of an adhesion molecule on the

surface of lymphocytes (L-selectin) is increased following an *in vitro* low temperature, long duration hyperthermia treatment (Evans *et al.*, manuscript submitted).

In our experiments, we find a greater number of NK cells in the tumor vasculature and in the tumor tissue immediately following WBH. Few NK cells are present in non-treated tumors, but a basal level of primary tumor control by NK-mediated apoptosis does exist. We have found that we can consistently suppress this basal level by administering continual injections of asialo GM-1 antibody and promote the growth of human tumors in our SCID model. Furthermore, tumor-bearing mice injected with asialo-GM1 antibody (SCID and Balb/c) have shorter survival times (data not shown). NK cells are surveillance lymphocytes that can potentially kill circulating tumor cells and metastatic foci (Gorelik *et al.*, 1982). The presence of a large number of NK cells in a primary tumor is uncommon, but they have been seen infiltrating metastatic melanoma in humans (Kornstein *et al.*, 1987). Also, adoptively transferred NK cells have been shown to be in direct contact with metastatic tissue and induce apoptosis of tumor cells (Okada *et al.*, 1996). The increase in tumor-cell apoptosis observed in this study could be explained by a greater number in NK cells at the site of tumor, as well as an increase in specific NK cell activity. It is possible that WBH increases the ability of NK cells to adhere to blood vessels and migrate into tumor tissue.

NK cell accumulation could also be caused by an increase in blood flo through the tumor following WBH. The enlarged appearence of blood vessels shown in the study support this possibility. There is a long-standing recognition that the intra-tumor pressures may serve to effectively block the entry of various types of therapies or immunoeffector cells (Jain, 1997). The application of WBH may allow allow for a sustained vasodilatation of the vessels within the tumor, reducing the intra-tumor pressure and allowing a greater delivery of immunoeffector cells into the tumor. As shown in this study, enlarged blood vessels are present in the colon 26 tumor up to the

time of resection (1-2 weeks post hyperthermia). This long term effect on the vasculature in the tumor are further suggestive of the development of conditions which are similar to those which occur in an inflammatory response.

The possibility that WBH causes changes in tumor cells that affect NK cell activity and/or recruitment, such as the expression of new molecules on the surface of the tumor, has not been addressed here. A study by Fujieda *et al.* (1995) has shown that heat shock enhances the susceptibility of tumor cells to lysis by lymphokine activated killer cells. Other recent studies by Multhoff *et al.* (1995a,b) suggest that the expression of a heat shock protein on the surface of tumor cells may serve as receptors for NK cell killing. In support of this possibility, we have found that the expression of several HSP's are significantly increased on human breast tumors following WBH (R.Burd, X.-Y. Wang, E. Repasky and J. Subjeck, manuscript in preparation). Other possibilities include alterations in MHC I which acts as killer inhibitory receptors (Moretta *et al.*, 1996 ).

While many questions are raised by this data which need to be addressed, these somewhat surprising effects of low temperature, (fever-range) WBH, strongly suggest that there is an anti-tumor immune effect, which if better understood, may be improved and exploited for therapeutic use. Particularly appealing is the possibility that this treatment may work well as a new adjuvant and applied with other therapies designed to stimulate the existant immune response, i.e., cytokine treatments, adoptive therapies, or gene-based therapies, all of which help to target the tumor by immune system- mediated cell killing. However, if we are simply mimicking the thermal element of a fever, perhaps this treatment is not "new" at all but in fact has been conserved through millions of years of evolution because of the potential benefit of transient hyperthermia to the immune response. Thus, it is important to define as soon as possible the various immunological parameters which may be affected by this treatment. For example, what

is the role of cytokines in the observed anti-tumor effects? Are there certain cytokines which are actually induced by the low temperature, long duration WBH which may help to stimulate the anti-tumor immune response? Will exogenous addition of cytokines be synergistic with mild WBH in the treatment of tumors? Further studies on the potential mechanism(s) of increased anti-tumor effects caused by low temperature, long duration (fever-range) whole body hyperthermia may lead to the development of this procedure as useful and safe addition to cancer therapy.

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## Figure Legends

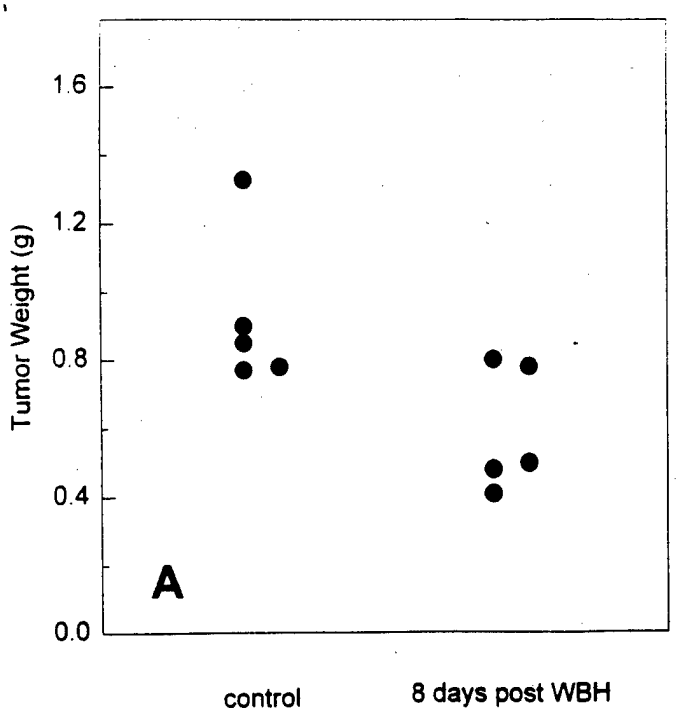
**Fig. 1a** Effects of WBH on the growth of a human breast tumor implanted into the abdominal fat pad of SCID mice. Eight days following WBH, tumors were excised and weighed. **b-c**, Evidence that NK cells mediate tumor reduction following WBH in SCID **(b)** and Balb/c mice **(c)**. SCID mice bearing a s.c. human breast tumor and Balb/c mice implanted s.c. with a syngeneic tumor, colon 26, were WBH treated with and without the depletion of NK cell activity. **(d)**, Effects of a second WBH treatment on the growth of an s.c. implanted breast tumor in SCID mice 6 days following the initial treatment. Data are +/- s.e.m. of 5 mice in each group.

**Fig. 2** NK cell mediated apoptosis is responsible for tumor volume reduction. Human breast tumors were implanted into the gonadal fat pad of SCID mice and apoptotic cells (arrows) were visualized using the TUNEL method. **(a)**, Tumor from a non-WBH treated mouse. **(b)**, Tumor from a mouse treated with WBH. **(c)**, Tumor from a mouse in which NK cell activity was depleted prior to WBH treatment. Eight hours following hyperthermia tumors were removed and paraffin sections were prepared for the TUNEL reaction. Sections were visualized using a fluorescence microscope. Magnification X 200, (inset X 600) bar indicates 100 μm.

**Fig. 3** Recruitment of immune cells to human breast tumors implanted in the gonadal fat pad of SCID mice following WBH. **(a),** Histological section of a tumor from a non-WBH treated mouse, and **(b),** a tumor from a WBH-treated mouse showing tumor destruction and the presence of an infiltrate (arrows) composed of NK cells, monocytes/macrophage, and granulocytes. Tumors were removed 8hr following WBH and paraffin sections were stained with hematoxylin and eosin. Magnification X600, bar indicates 30 μm. **(c),** Fluorescent staining of murine NK cells (arrows) in a tumor from a non-WBH treated mouse, and **(d),** a tumor from a WBH-treated mouse. Tumors were removed 8hr following WBH and Cryostat sections were stained with asialo GM1-FITC as

described. Sections were then viewed using a confocal microscope magnification 600X. **(e)**, Fluorescent identification of DiI labeled human NK cells (arrows) in a tumor from a non-WBH treated mouse, and **(f)**, a tumor from a WBH treated mouse. DiI labeled human NK cells were injected 24hr before WBH. Immediately following the treatment, the tissue was placed in Tissue Tek O.C.T. compound and frozen. Cryostat sections were observed using a fluorescent microscope. Magnification X1000, bar indicates 30 μm.

**Fig. 4** Enlargement of tumor blood vessels following WBH. **(a)**, Colon-26 tumor from a non-treated mouse showing normal blood vessels (arrows). **(b)**, Colon-26 tumor from a WBH treated mouse showing enlarged blood vessels (arrows). Two weeks after WBH tumors were removed and paraffin sections were stained with hemotoxylin and eosin. Magnification X 200, bar indicates 100 μm.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1.2
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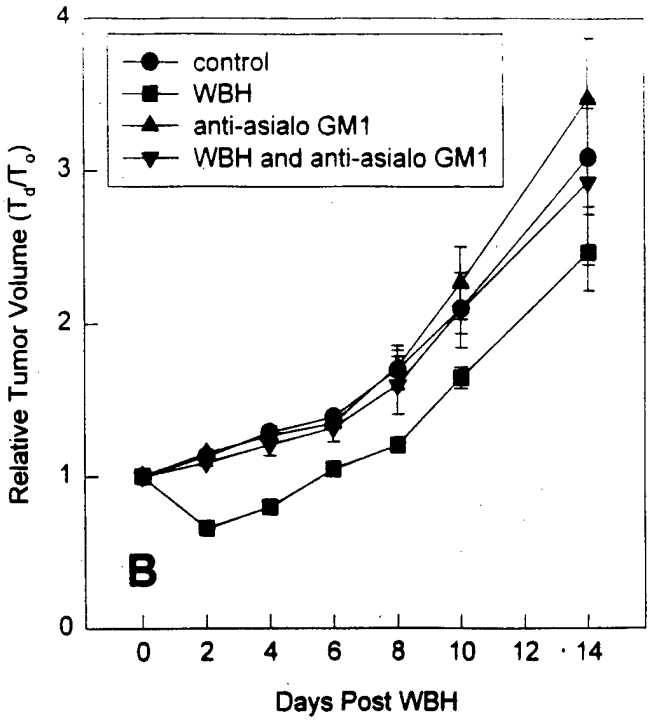
0.4
0.0
A
control
8 days post WBH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WBH
anti-asialo GM1
WBH and anti-asialo GM1
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3
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1
0
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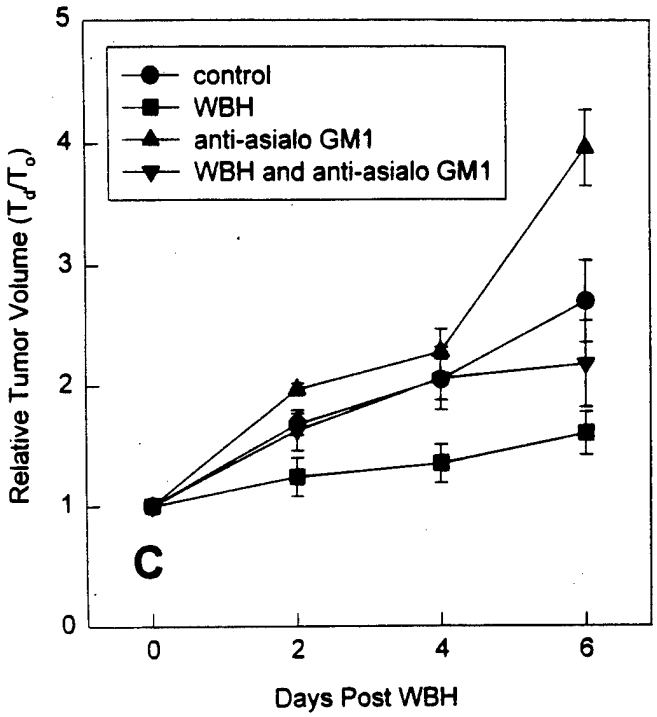
0
2
4
6
8
10
12
14
Days Post WBH


control
WBH
anti-asialo GM1
WBH and anti-asialo GM1
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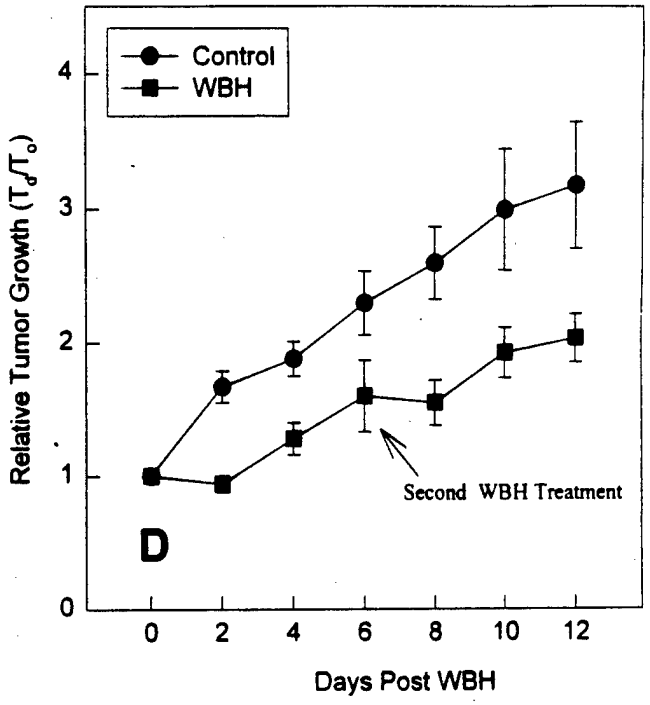
3
2
1
0
C
0
2
4
6
Days Post WBH


Control
WBH
Relative Tumor Growth ($T_d/T_o$)
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Second WBH Treatment
D
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2
4
6
8
10
12
Days Post WBH

**Fig. 1**

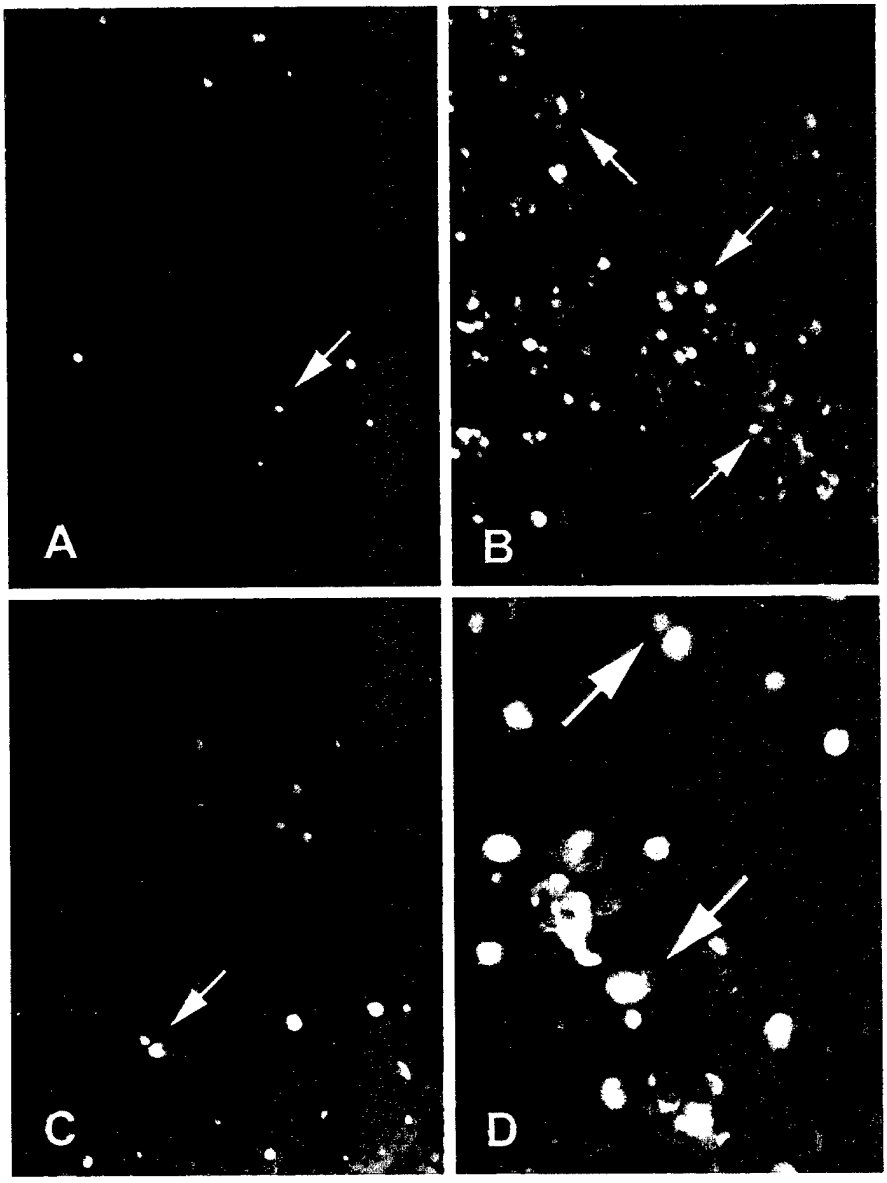


Fig. 2

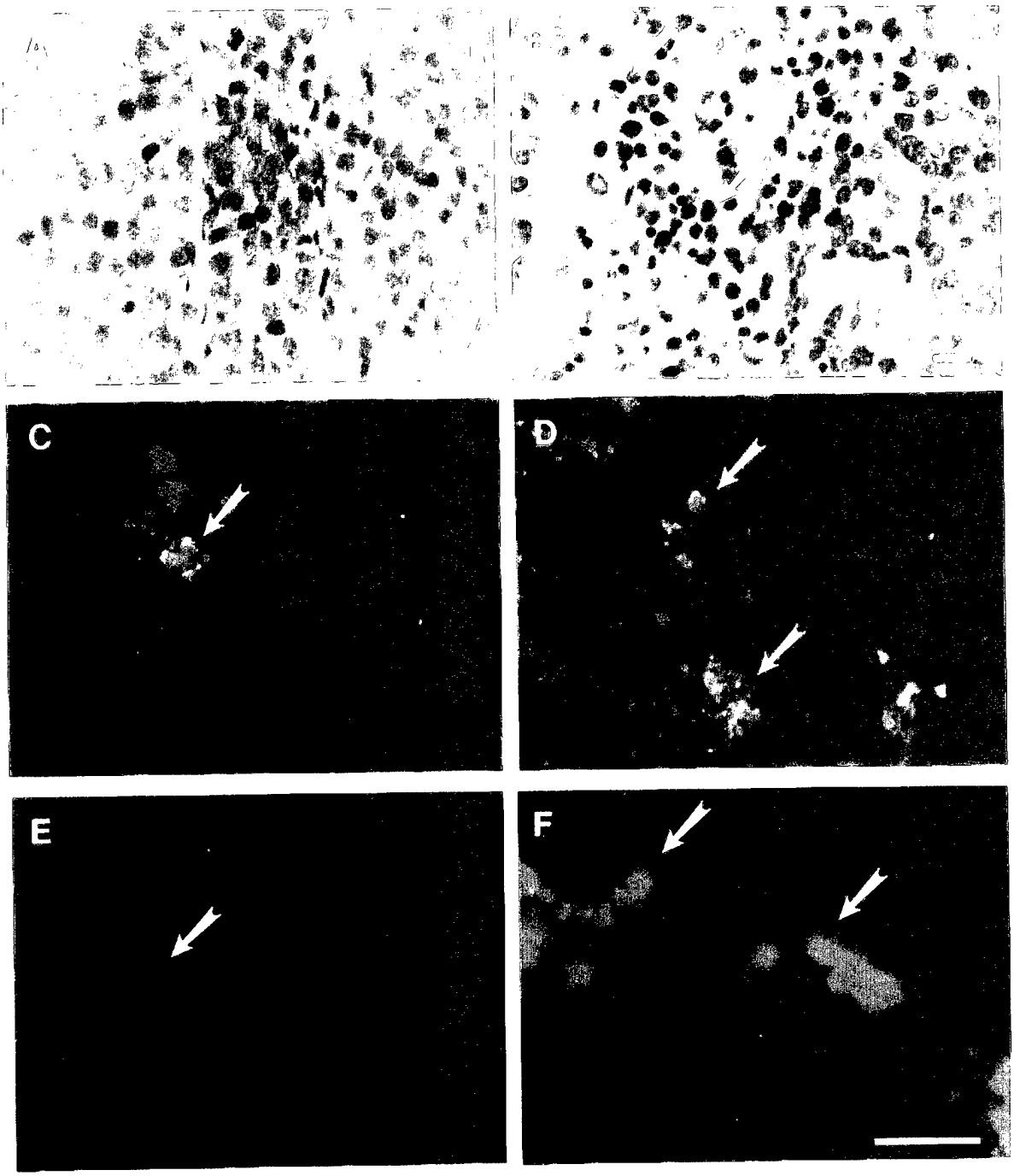


Fig. 3

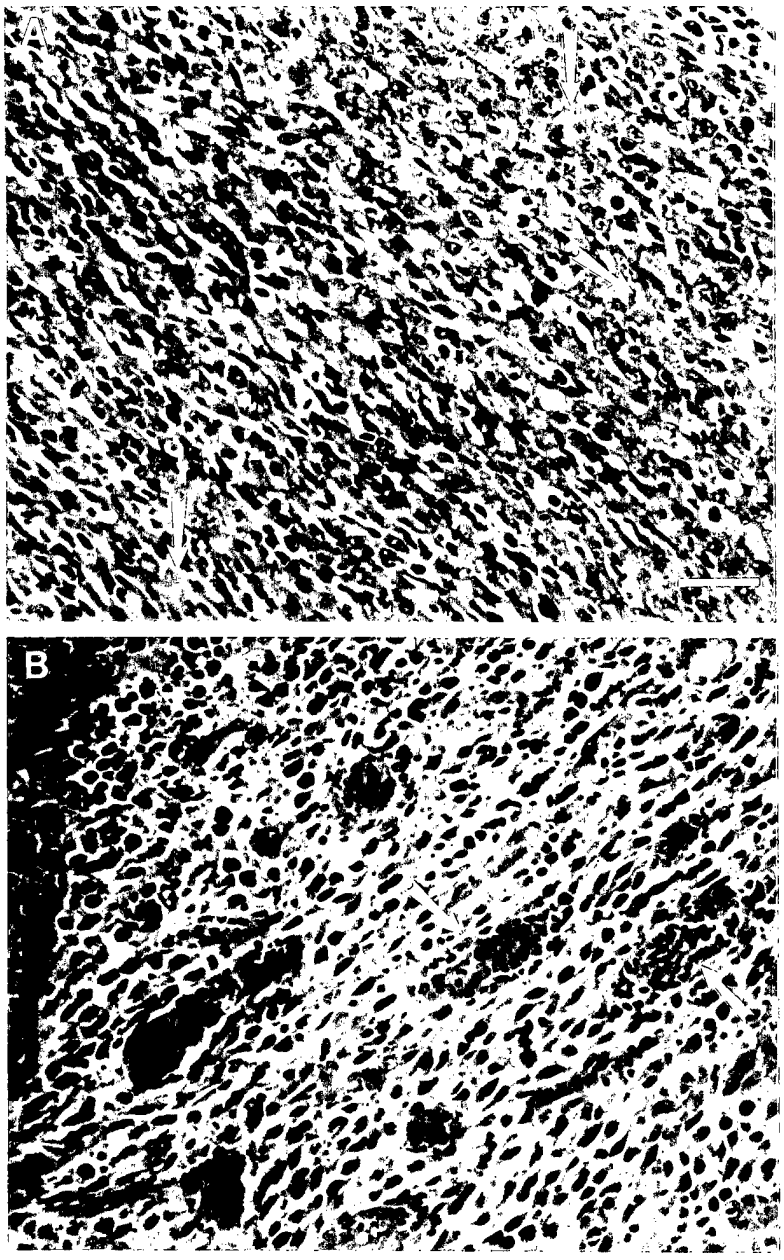


Fig. 4