Behavior of Metastatic and Nonmetastatic Breast Tumors in Old Mice

CLAUDIA GRAVEKAMP,*[,1] ROZA SYPNIEWSKA,* SUSAN GAUNTT,* MELISSA TARANGO,* PAUL PRICE,* AND ROBERT REDDICK†

*University of Texas Health Science Center at San Antonio, *Department of Cellular and Structural Biology, San Antonio, Texas 78245; and †Department of Pathology, San Antonio, Texas 78229*

Breast cancer incidence and mortality increase with age. A better understanding of the biological behavior of metastatic and nonmetastatic breast tumors in older subjects may help to develop improved breast cancer therapies. In this study, we used syngeneic metastatic (4TO7cg) and nonmetastatic (64pT) mouse breast tumor models at three age levels to evaluate various characteristics that are considered to be important for effective anti-breast cancer immunotherapy. These included tumor size and growth, metastases, vascularization, gene expression levels of the tumor-associated antigen (TAA) Mage-b (homologous to human MAGE-B) in primary breast tumors and metastases, and the presence of $CD4^+$ and $CD8^+$ T cells in the inguinal lymph nodes at the site of the tumor. The primary breast tumors and metastases were generated by injection of mouse mammary tumor cell lines 4TO7cg or 64pT into a mammary fat pad of normal 3-, 9-, or 21/24-month old BALB/c mice. In the nonmetastatic breast tumor model, significantly smaller tumors were observed in old compared with young mice. This was associated with a significant increase in the percentage of $CD8^+$ T cells in inguinal lymph nodes and significantly higher Mage-b expression levels in the primary tumors at old age. In the metastatic (4TO7cg) breast tumor model, a less pronounced, not statistically significant, smaller tumor size was found in the old mice, without a difference in the percentage of $CD8^+$ T cells or Mage-b expression levels. However, in this mouse model almost all metastases showed high levels of Mage-b expression (2- to 3-fold higher than the primary tumors in the same animals) regardless of age. These results indicate that the metastatic and nonmetastatic breast tumor models could be useful model systems to analyze how breast cancer vaccines for humans can be tailored to old age. Exp Biol Med 229:665–675, 2004

This work was supported by the American Federation of Aging Research (AFAR) grant A000106, the Barbara Bowman Award in Cancer Prevention Research from the San Antonio Cancer Institute (SACI), the Helen Freeborn Kerr grant, and the National Institute of Aging (NIA) training grant 5T32AG000205-09.

[1] To whom correspondence should be addressed at University of Texas Health Science Center at San Antonio, Department of Cellular and Structural Biology, 14960 Omicron Drive, San Antonio, TX 78245. E-mail: cgraveka@idd.org or gravekamp@uthscsa.edu

Received March 15, 2004.
Accepted March 19, 2004.

1535-3702/04/2295-0001$15.00

Key words: metastatic breast tumor model; nonmetastatic breast tumor model; Mage-b expression; aging; 64pT; 4TO7cg

Breast cancer is the second leading cancer in the United States, with 193,700 new cases diagnosed in 2001 (1). More than half of new breast cancer cases occur in women older than 65 years (2). Hence, there is an increasing need to optimize breast cancer management and therapy for elderly patients. Both metastatic and nonmetastatic breast cancers occur frequently in the elderly (3, 4). As in younger individuals, metastatic breast cancer has a poor prognosis. It has been shown that 80% of breast cancers metastasize to the bones, 25% to the lungs, 22% to the liver, 15% to the brain, and 22% to other organs (5).

Although first-line endocrine therapy with tamoxifen or the newer third-generation aromatases is promising (3), metastatic breast cancer is difficult to control (6). Moreover, aggressive treatment using radiation or chemotherapy is not a good option for elderly women, because they are generally more subject to frailty. In this respect, enhancement of specific helper and cytotoxic T lymphocyte (CTL) responses to breast tumors through vaccination could potentially lead to specific elimination of micrometastases and/or residual tumor cells. A better understanding of the biological behavior of primary and metastatic breast tumors in older subjects may help in the development and optimization of such immunotherapies.

Results of past studies generally indicate that naturally occurring tumors in humans show slower growth with advancing age and longer host survival (7, 8). A slower progression of tumors in old hosts has also been observed in animal models such as the B16 melanoma model (7), the EMT6 lung tumor model (9), and the AKR lymphoma model (10). If, as has been suggested (11, 12), age-acquired immune deficiency is related to the higher incidence of cancer at old age, these findings are somewhat counter-intuitive. T-cell unresponsiveness, known to be associated with old age (11), should contribute to larger, rather than smaller, tumors at old age. A decrease in the number of naive T cells at older age is considered to play an important

role in the age-related decline in T-cell responses (13). This could be responsible for the well-described decline in vaccine responsiveness in elderly patients (14, 15). Possible explanations for the observed slower tumor growth at old age could be increased replicative senescence, reduced angiogenesis, lower levels of expression of growth factors/receptors, or tumor-associated antigens at the cell membrane (7).

T-cell unresponsiveness also occurs in cancer patients, although this differs from the age-related T-cell unresponsiveness in the elderly. In cancer patients, CTLs are often found at the site of the tumors. However, they have not been shown to destroy tumor cells (16). Multiple causes have been considered to account for the unresponsiveness of the CTL in malignant neoplasms (17). Most frequently described is the low expression of major-histocompatibility complex (MHC) molecules (required for antigen recognition by T cells) and co-stimulatory molecules (required for T-cell activation) at the cell membrane of primary and metastatic tumors in humans and mice (18–20). In addition to MHC and co-stimulatory molecules, expression of tumor-associated antigens (TAAs) is important for activation of tumor-specific T cells.

In a study with human breast tumors, expression of the TAA MAGE was detected as frequently as in 92% of the cases (21). MAGE peptide-based vaccines have been used in clinical trials with melanoma patients, but with limited success (22–24). However, a suitable mouse tumor model that would permit the optimization of MAGE-encoding cancer vaccines in aged mice is currently not available. Therefore, we have studied various mouse breast tumor models for the expression of mouse TAA Mage that is homologous to human MAGE (25, 26). Recently, we characterized the expression of Mage-b3 in the breast tumors of MMTV-v-Ha-ras and MMTV-c-myc transgenic mice (27). In addition, we identified Mage-b1/2 in breast tumors of a metastatic (4TO7cg) and a nonmetastatic (64pT) mouse model (28).

In this present study we used the 4TO7cg and 64pT mouse breast cancer models to obtain information on their potential usefulness to test MAGE-encoding cancer vaccines in metastatic and nonmetastatic breast cancer at old age. We studied various characteristics that are important for vaccine therapies in the elderly. We focused on tumor size and growth, the presence of $CD4^+$ and $CD8^+$ T cells at the site of the tumor, and expression levels of the Mage-b TAA in primary breast tumors or metastases in relation to aging. Two important findings were obtained. First, the results basically confirm the reported reduced tumor growth at old age. Second, high Mage-b expression levels were found in primary tumors and metastases at old age in association with the presence of $CD8^+$ T cells at the site of the tumor. These results indicate that the breast tumor models of this study are useful to test and improve Mage-b vaccines at old age. Ultimately, these models may teach us how to improve human MAGE vaccines for elderly breast cancer patients.

Materials and Methods

Mice. Normal BALB/c female mice of 3, 9, and 21 or 24 months were obtained from the National Institute of Aging/Harlan (Bethesda, MD) and maintained in the animal facility of the University of Texas Health Science Center, San Antonio, according to the Association and Accreditation of Laboratory Animal Care (AACAC) guidelines. Mice of different ages were from the same colony and arrived at our facility 1 week prior to the experiments. In the experimental mice that received tumor cells, at least 10 mice per age group (3, 9, and 21/24 months) were used; in the control mice that did not receive tumor cells, only five mice per age group (3, 9, and 21 months) were used. The maximum life span of female BALB/c mice is 33.5 months, and the mean life span 25.1 months (29). Mice of three different ages that are important for vaccine studies in the future were selected. Three months was selected as the age at which a proper immune response could be expected (positive control), 9 months as the intermediate age, and 21 or 24 months as the old age at which T-cell unresponsiveness could be expected (13).

Cells and Cell Culture. The mouse mammary tumor cell lines 64pT and 4T07 were kindly provided by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI). The 64pT breast tumor is a spontaneous fusion between mammary cell lines 4TO7 and 68H (30). These cell lines were developed from mammary tumors of BALB/c mice infected with the mouse mammary tumor virus (MMTV). The 64pT tumor-cell line is nonmetastatic. The original 4TO7 tumor cell line produces pulmonary metastases when injected intravenously (31, 32). After culturing the 4TO7 tumor cell line for about 1 year in our laboratory, it had metastatic capabilities when injected into mammary fat pads (this line denoted as 4TO7cg). The mammary tumor cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1 mM mixed nonessential amino acids, 2 mM L-glutamine, insulin (0.5 HSP units/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml).

Mammary Tumors and Metastases. The injection of 100 μl of DMEM with various concentrations of viable 64pT or 4TO7cg cells into a mammary fat pad resulted in a breast tumor at the place of injection, designated as the primary tumor, and metastases in normal BALB/c mice within 2–4 weeks. Palpable tumors were measured in two perpendicular axes with tissue calipers. Mean tumor growth (TD) was calculated as the square root of the product of two perpendicular tumor diameters measured with a caliper. The mice were sacrificed and tumors were dissected, measured, and weighed 29, 49, or 69 days after injection of 10^5, 10^4, or 10^3 64pT or 4TO7cg cells. From each tumor, one third was kept in 10% formalin for 48 hrs and then stored in 70% ethanol until processed for histology. The remaining two thirds of the tumor was snip frozen in liquid nitrogen and then stored at –80°C for reverse-transcriptase polymerase

chain reaction (RT-PCR). Metastases were visible as nodules on the surface of the lungs, liver, and diaphragm, and in the peritoneal cavity. The number of mice with and without metastases was recorded. In some cases, a mouse died during anesthesia. With the 64pT model, three mice died before final analysis, and with the 4TO7cg model, one mouse died before final analysis.

Histological Analysis. Tissue samples were fixed in neutral buffered formalin and 48 hrs later placed in 70% ethanol. The tissue samples were embedded in paraffin, and 5-μm thick sections were cut from each block. The sections were stained with hematoxylin and eosin (H&E) and examined using light microscopy.

RT-PCR. RNA was isolated according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Conversion of 1 μg of mRNA into cDNA was performed with the Superscript Preamplification system (Life Technologies). Subsequently, 10 μl of the cDNA was amplified by hot-start PCR (Platinum PCR SuperMix, Life Technologies; 40 cycles at 94°C for 30 secs, 50°C for 30 secs, and 72°C for 2 mins) in a thermocycler from Perkin-Elmer (Norwalk, CT). Primers F111 and R1182: 5′ TCA ACT ACA CAT TAG AGG ACT T were generated to obtain a 1071 bp product, including the complete orf of Mage-b1, -b2, or -b3 (NCBI data bank AY196960; Ref. 28). To determine the quality of the RNA samples and to detect possible contamination of genomic DNA in the RNA samples, all tissue samples were tested by RT-PCR using β-actin primers: 5′ TCA TGA AGT GTG ACG TTG ACA TCC GT 3′ and β-actin 5′ CCT AGA AGC ATT TGC GGT GCA CGA TG 3′ (Life Technologies). Contamination of genomic DNA in the RNA samples should be detected with these primers because two bands of 285 bp (mRNA) and 408 bp (genomic DNA), instead of one, will appear in the agarose gel after RT-PCR. Both primers anneal in different exons and will amplify a product containing an intron of 123 bp from genomic DNA, which is excised from mRNA. In addition, all samples were subjected to RT-PCR without the enzyme RT.

Southern Blot Analysis. RT-PCR products were separated in an ethidium bromide–stained agarose gel and transferred to an Immobilon-N^+ membrane (Amersham, Buckinghamshire, England) and hybridized with a chemiluminescence-labeled Mage-b–specific probe (enhanced chemiluminescence; Amersham) according to the manufacturer's instructions. The Mage-b–encoding DNA probe was obtained by PCR from the Mage-b–encoding vector (NCBI data bank AY196960; Ref. 28), and specificity was confirmed by DNA sequencing.

Comparison of Expression Levels of Mage-b Between Young and Old Mice. In order to compare Mage-b expression levels in breast tumors and metastases of young with old mice, the RT-PCR and gel electrophoresis procedures, as well as exposure times, were standardized. For each individual sample, β-actin was used as an internal reference. Testis was used as a positive control for Mage-b in each gel. To eliminate experimental variation in Mage-b expression levels, samples of mice of various ages were tested in the same RT-PCR experiment, and PCR products were analyzed in the same agarose gel and southern hybridization. From each sample, 200 ng RNA was used for RT-PCR, and 20 μl of Mage-b–specific RT-PCR product was loaded on the agarose gel. After southern blotting, each film was exposed for 1 min. Mage-b expression levels were measured by a densitometer using a Gel Doc 2000 (Bio-Rad, Glercules, CA).

Statistical Analysis. To determine if differences in tumor weight, percentage of T cells, or Mage-b expression levels were statistically different, the Wilcoxon rank sum test, analysis of variance (ANOVA) test, unpaired F test, or the unpaired t test was used when appropriate, with $P < 0.05$ being considered significant.

CD8 and CD4 Staining. Cells were isolated from inguinal lymph nodes at the site of the tumor according standard protocols (33) and directly analyzed (without any *in vitro* stimulation) for the presence of $CD4^+$ and $CD8^+$ T cells. To this aim, 10^6 cells were pre-incubated with 0.5 μg of rat anti-mouse Fc blocker (CD16) monoclonal antibody in order to prevent binding of the Fc domain of non–antigen-specific antibodies to type II and III/Fc receptors, followed by incubation with 0.5 μg fluorescein isothiocyanate (FITC) conjugated rat-anti-mouse CD8 monoclonal antibody and 0.5 μg of R-phycoerythrin (R-PE) conjugated rat anti-mouse CD4 monoclonal antibody. Wash steps occurred between each incubation with PBS containing 1% FBS, except after the Fc blocker. Isotype controls to identify binding of nonspecific antibodies to the CD4 (IgG2b) or CD8 (IgG2a) were tested and appeared to be negative. All antibodies were purchased from Pharmingen (San Diego, CA). All cells were analyzed by a fluorescent activated cell sorter (FACS). Each mouse was individually analyzed. The results were averaged, and the possible difference in the percentage of $CD4^+$ or $CD8^+$ T cells between young and old mice was determined by statistical analysis.

Results

Metastatic and Nonmetastatic Tumor Behavior at Young and Old Age. To induce tumors in the young or old BALB/c mice, syngeneic 64pT or 4TO7cg cells were injected in a mammary fat pad. To determine the optimal cell numbers, 10^3, 10^4, or 10^5 of the nonmetastatic 64pT mammary tumor cell line were injected into a fat pad of normal BALB/c mice of different ages (3, 9, or 24 months). Sixty-nine, 49, or 29 days later the mice were sacrificed and analyzed for the following parameters: tumor size/weight, latency, vascularization of primary tumors, and frequency of metastases. In addition, primary tumors were histologically analyzed. The results indicated that although injection of 10^3 64pT tumor cells resulted in too few tumors, 10^5 64pT tumor cells resulted in early death. Injection of 10^5 4TO7cg cells resulted in early death as well (data not shown).

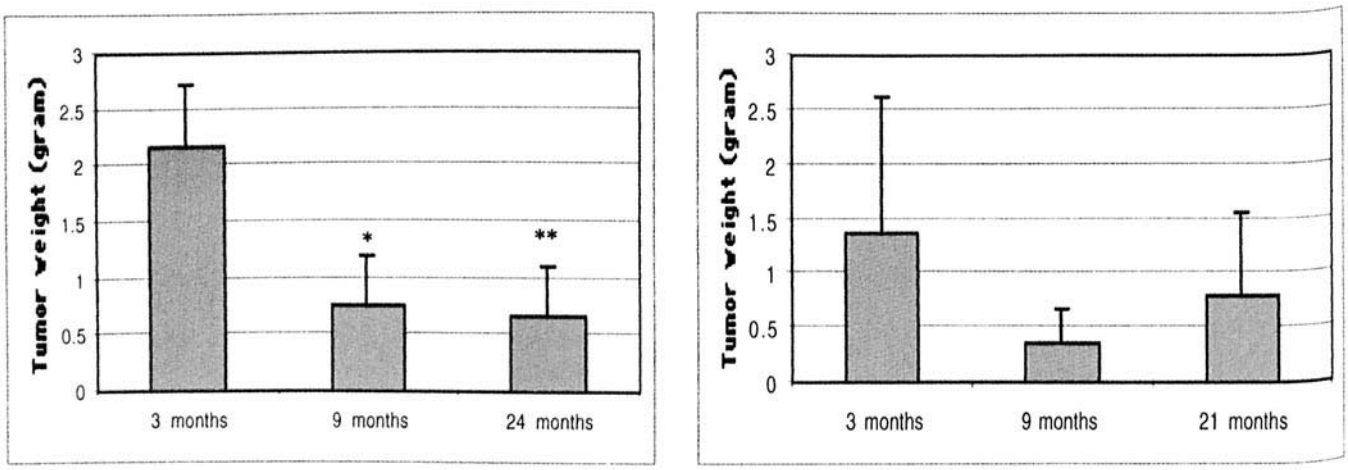

Figure 1. The effect of aging on the growth of 64pT (A) or 4TO7cg (B) primary breast tumors in immune competent BALB/c mice of various ages. The number of mice per group is as follows: 64pT model, $n = 10$ for all ages; 4TO7cg model, $n = 10$ for 3 and 9 months, and $n = 20$ for 21 months. For comparison of tumor weight in the specific age groups, a Tukey-Kramer multiple comparison test was used. The weight of 64pT tumors was significantly higher in the mice of 3 months compared with those in 9-month-old mice (*, $P < 0.01$) or 24-month-old mice (**, $P < 0.01$), but no difference in weight of 64pT tumors was observed in mice of 9 compared with 24 months ($P > 0.05$). No statistical difference was observed in the weight of 4TO7cg tumors in mice of different ages ($P > 0.05$). The error bars represent the standard deviation.

Therefore, we considered 10^4 as the highest possible tumor cell number to test for both cell lines.

With 10^4 64pT cells, significantly smaller primary breast tumors were observed in old than in young mice (Fig. 1A). With 10^4 of the metastatic 4TO7cg tumor cells, essentially the same results were obtained, but the age-related difference was less pronounced and not significant (Fig. 1B). In contrast to the 64pT model, numerous metastases developed in the 4TO7cg mouse model. These 4TO7cg-derived metastases were predominantly found at the surface of the lungs (range, 1–3 metastases); in the peritoneal cavity (range, 2–15 metastases); and sporadically at the surface of the liver (range, 1 metastasis) or on the diaphragm (range, 1–2 metastases; Table 1). Although a 3-fold increase was observed in the number of mice with metastases at the surface of the lungs or in the peritoneal cavity in old mice compared with young mice, this was statistically not significant.

Considerable variation in individual tumor sizes was observed in both metastatic and nonmetastatic mouse models. We compared the standard deviation of tumor size at each individual time point (days after injection with tumor cells) of mice at three different ages within each breast tumor model. In the 4TO7cg model, the variation in tumor size was large independently of age. In the 64pT model, the variation in individual tumor size was significantly greater in older mice (24 months) than in younger ones (3 months) at later stage of tumor growth (days 40 and 43 after tumor cell injection; F test, unpaired, $P < 0.05$; Fig. 2). However, in both models significant differences in variation of tumor size was observed at early stage of tumor growth. These differences might reflect tumor size–related differences, rather than variation in tumor size within the model.

Table 1. Effect of Aging on Metastatic Breast Cancer in the Mouse

Characteristics of mice injected with 4TO7cg tumor cells	3 months	9 months	21 months
Vascularization of tumors	+++++	++	+
No. with primary breast tumors/total	6/10	5/10	14/19
No. with lung metastases/total	1/10	1/10	6/19
No. with metastases in peritoneal cavity/total	1/10	0/10	6/19
No. with metastases on diaphragm/total	1/10	1/10	0/19
No. with liver metastases/total	0/10	1/10	0/19
Characteristics of mice without 4TO7cg tumor cells			
No. with spontaneous tumors in peritoneal cavity/total	0/5	0/5	3/5
No. with spontaneous tumor on liver/total	0/5	0/5	1/5

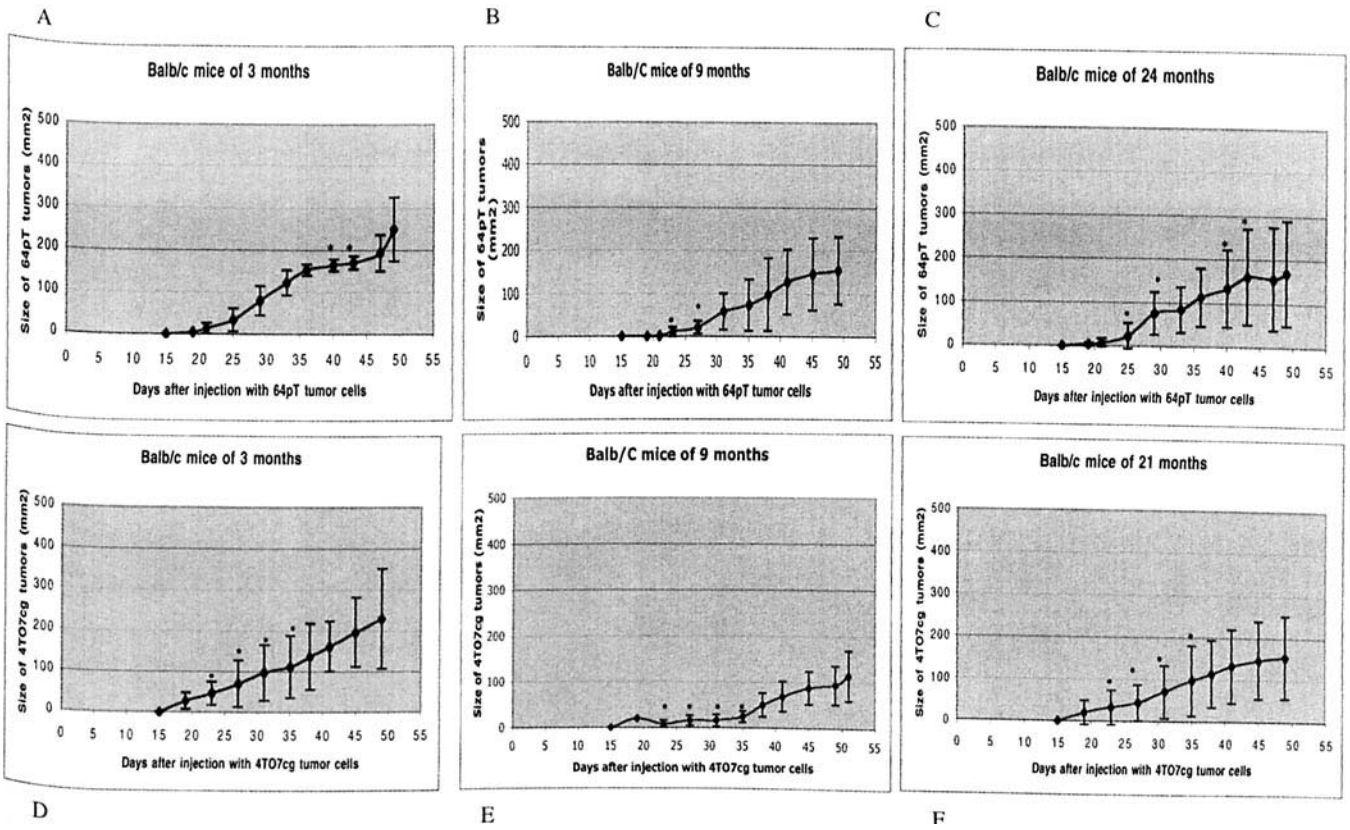

Figure 2. Variation in the size of 64pT primary breast tumors in mice of 3 (A), 9 (B), and 24 (C) months, or 4TO7cg primary breast tumors in 3 (D), 9 (E), and 21 (F) months. The error bars represent standard deviation. Statistical differences were observed in variation of tumor sizes at the later stage of tumor growth (*) in the 64pT model (*F* test, unpaired, $P < 0.05$). However, significant tumor size–related differences, rather than variation in tumor sizes, were observed at the early phase of tumor growth (·) in both breast tumor models (*F* test, unpaired, $P < 0.05$).

No significant difference between young and old mice was observed in the frequency (Tables 1 and 2) or latency (data not shown) of the 64pT and 4TO7cg breast tumors.

Vascularization of the 4TO7cg tumors was more developed in the young mice than in the older ones (Table 1). This difference was less pronounced for the 64pT tumors (Table 2). Vascularization was more prominent in the breast tumors of the metastatic 4TO7cg than the nonmetastatic 64pT model, which is not surprising because angiogenesis is required for invasive tumor growth and metastasis (34). No obvious histological differences were observed between the primary breast tumors of mice of various ages (data not shown). The tumors consisted of pleomorphic cells with oval- to round-shaped nuclei. Mitotic activity was prominent. Large areas of necrosis were present in most tumors. No organoid areas were identified. In some tumors, normal breast tissue was present adjacent to the tumor site. Time curves of development of individual primary tumors in mice of various ages showed that differences in tumor size were most pronounced during the first 29 days after injection of the tumor cells (data not shown), suggesting that this earlier time point may be most suitable for histological analysis. However, histological differences were found between lung metastasis in young and old mice. This was not the case for other types of metastases.

Percentage of $CD8^+$ and $CD4^+$ T Lymphocytes at Various Ages. In view of the reported T-cell unresponsiveness at old age, it was deemed important to assess whether $CD8^+$ and $CD4^+$ T lymphocytes were associated with tumor formation in young and old mice.

Table 2. Effect of Aging on Nonmetastatic Breast Cancer in the Mouse

Characteristics of mice injected with 64pT tumor cells	3 months	9 months	24 months
Vascularization of the 64pT tumor	+++	++	++
No. of mice with primary breast tumors/total No. of mice			
10^3 64pT tumor cells injected into mammary fat pad	2/10	2/10	1/7
10^4 64pT tumor cells injected into mammary fat pad	3/6	6/9	6/7
10^5 64pT tumor cells injected into mammary fat pad	5/9	9/10	7/9

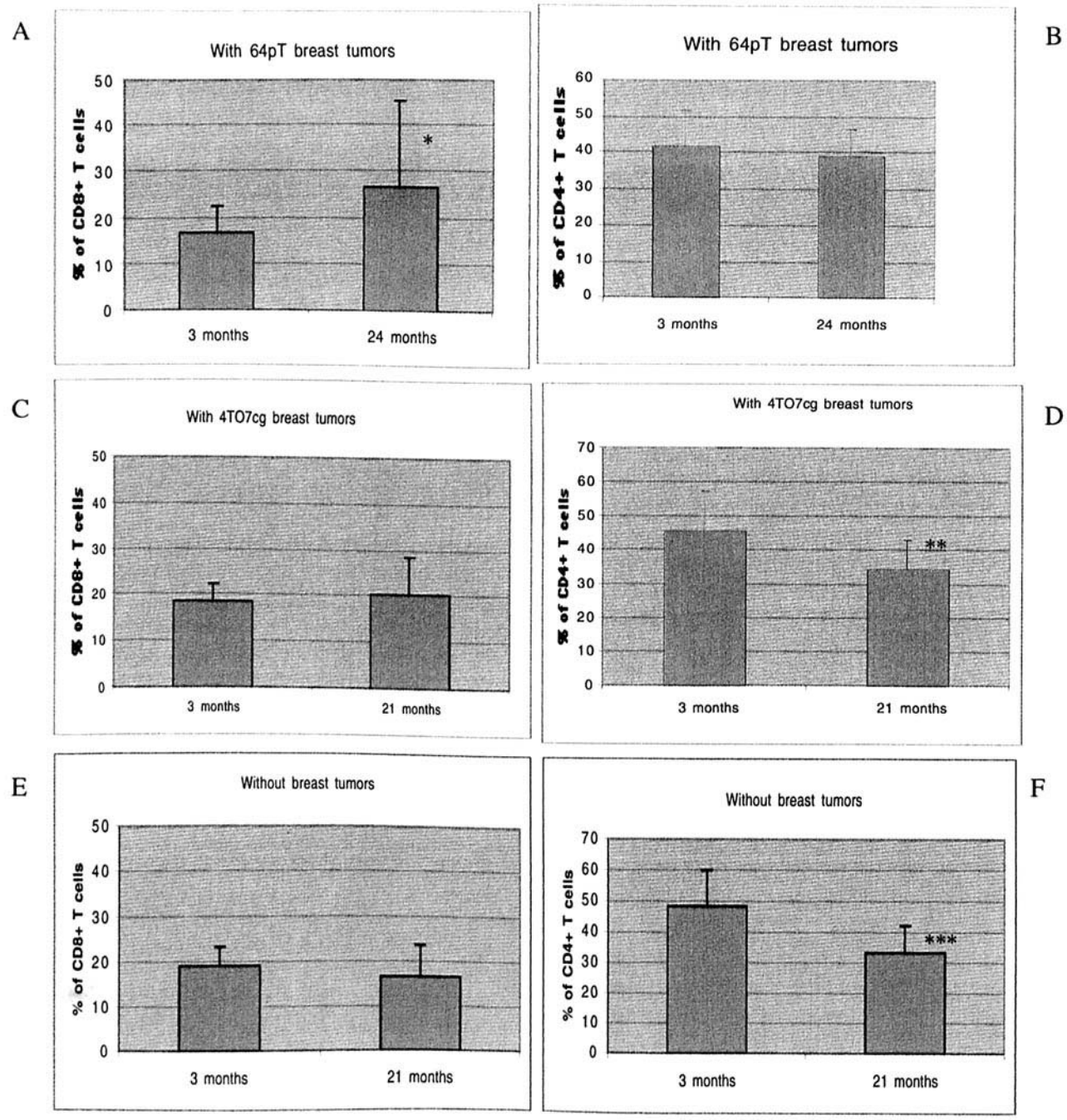

Figure 3. The effect of aging on T cells. The effect of aging was measured on $CD8^+$ T cells in inguinal lymph nodes of BALB/c mice with 64pT tumors (A), 4TO7cg tumors (C), or of noninjected BALB/c mice (D), as well as on $CD4^+$ T cells in inguinal lymph nodes of BALB/c mice with 64pT tumors (B), 4TO7cg tumors (D), or of noninjected BALB/c mice (F). CD4 and CD8 receptors were measured on T cells isolated from inguinal lymph nodes of individual mice of 3 and 24 months. The results were averaged per age group and subjected to statistical analysis. For comparison of the percentage of T cells in the different age groups, an unpaired *t* test was used. Significance was at the $P < 0.05$ level. The following *P*-values were found: 64pT model/$CD8^+$ T cells, $P = 0.0379$ (*); 64pT model/$CD4^+$ T cells, $P = 0.9621$; 4TO7cg model/$CD8^+$ T cells, $P = 0.5187$; 4TO7cg model/$CD4^+$ T cells, $P = 0.0051$ (**); noninjected BALB/c mice/$CD8^+$ T cells, $P = 0.5242$; noninjected BALB/c mice/$CD4^+$ T cells, $P = 0.0488$ (***). The error bars represent the standard deviation.

Since the mice of 9 months behaved immunologically like mice of 3 months, we did not include the mice of 9 months in Figure 3. Somewhat surprisingly, in the nonmetastatic 64pT model, a significant increase in the percentage of $CD8^+$ T lymphocytes was observed in the draining inguinal lymph nodes of old compared with young mice (Fig. 3A). In the group of older mice, one lymph node exhibited a considerable higher percentage of $CD8^+$ T cells (86%) than the other lymph nodes (average 22%). This particular mouse had a tumor (weight 1.3 g) with lesions to the outside world. This one lymph node was responsible for the large standard deviation in Figure 3A. The increase in the percentage of

$CD8^+$ T cells was tumor-related because it was not found in mice without tumors, in which a slight (not statistically significant) decrease in the percentage of $CD8^+$ T cells was observed in old mice compared with young ones (Fig. 3E). Also in the metastatic 4TO7cg model, a slight increase in the percentage of $CD8^+$ T cells was observed in older mice compared with younger ones, although this was not statistically significant (Fig. 3C). In mice without tumor cell injection, a significant decrease in the percentage of $CD4^+$ T lymphocytes was observed in the inguinal lymph nodes of old compared with young mice (Fig. 3F). Such a decrease was also observed in the older mice with 64pT (statistically not significant) and 4TO7cg (statistically significant) breast tumors (Figs. 3B and 3D).

Mage-b Expression Levels in Tumors and Metastases at Various Ages. Expression level of the TAA, Mage-b, is likely to be an important determinant of a potential age-related loss in antitumor immunity. Mage-b expression levels in the metastatic and nonmetastatic breast tumors, as well as in the metastases, were determined by RT-PCR and Southern blotting. In the nonmetastatic model, the 64pT tumors showed a significantly higher Mage-b expression level in old than in young mice (Fig. 4A). In contrast to the nonmetastatic model, the metastatic 4TO7cg tumors showed a significantly lower Mage-b expression level in older mice than in younger ones (Fig. 4B). The Mage-b expression levels were considerably higher (4-fold) in the 64pT than in the 4TO7cg tumors. Most importantly, almost all metastases showed high expression levels of Mage-b (2- to 3-fold higher than in the 4TO7cg primary tumors), independent of age (Fig. 4C). A representative example of Mage-b expression in the primary breast tumors and metastases of mice of various ages is shown in Figure 5A and B.

Discussion

With the rapid increase in elderly cancer patients (1), it is well recognized that prevention and therapy need to be tailored to old age. Cancer vaccines are potentially a good candidate for cancer prevention and therapy in the elderly since it is relatively benign and therefore more applicable to frail individuals. However, model systems that allow preclinical development and optimization of immunotherapeutic approaches are currently not available. The main purpose of this study was to evaluate whether the metastatic (4TO7cg) and nonmetastatic (64pT) mouse syngeneic breast tumor models are useful for testing and optimization of breast cancer vaccines at old age. For this purpose, we examined various characteristics that are considered to be important for immunotherapy, such as tumor growth, vascularization, frequency of metastases, and the presence of $CD4^+$ and $CD8^+$ T cells at the site of the tumor, as well as expression of TAA Mage-b in relation to aging.

Some of our results are in agreement with the literature. For example, we confirm that tumors grow less aggressively in old as compared with young mice, a difference that is already apparent as early as 9 months of age. This slower growth of the injected tumor cells is likely due to the extracellular, aged environment, which may contain less growth factors and possibly promotes replicative senescence or differentiation of tumor cells. The possibility that a decline in ovarian function would cause the slower growth in older animals is refuted by the absence of estrogen receptors on the tumor cells.[1]

The effect of aging was more pronounced in the nonmetastatic model than in the metastatic model. Obvious was the greater variability in tumor sizes of 64pT breast tumors in older mice (9 and 24 months) compared with younger mice (3 months). Although we have no ready explanation for this phenomenon, it should be noted that most variables studied with age show increased individual variability, and it is possible that increased stochastic noise increases local variation of tumor growth. Others found that breast cancer appears biologically less aggressive at old age, but that older women have a greater frequency of differentiated tumors (35). In the metastatic 4TO7cg model, the variation in tumor size was high regardless of age. It is tempting to speculate that this is inherent to the specific biological attributes in which the 4TO7cg model distinguishes itself from the nonmetastatic 64pT.

We found a 3-fold higher number of mice with metastases at the surface of the lungs at older compared with younger age (Table 1). However, it has been shown that female BALB/c mice may develop peribronchial lymphatic nodules or bronchoalveolar adenocarcinoma at older age (36). Hence, the observed increase may not be caused by 4TO7cg-induced metastases (Table 1). However, our control mice (uninjected mice) did not show lung metastases. We also found a 3-fold higher number of mice with metastases in the peritoneal cavity of older mice compared with younger mice, whereas in the control group three out of five mice showed spontaneous tumors in the peritoneal cavity (Table 1). Hence, the possibility cannot be excluded that a mixture of spontaneous, age-related, BALB/c-derived and 4TO7cg-derived tumors/metastases in the peritoneal cavity and lungs was observed. For possible vaccine studies, the presence of spontaneous BALB/c-derived and 4TO7-derived tumors/metastases is interesting because both types of tumors/metastases expressed Mage-b (results not shown).

Another factor that may play a role in tumor growth is T-cell unresponsiveness, a well-described phenomenon in the elderly and in cancer patients (34). T-cell unresponsiveness should favor tumor growth because T cells are considered to play an important role in antitumor reactions. Hence, this phenomenon would not explain the slower

[1] Dr. F. Miller, Karmanos Institute, Detroit, Michigan, personal communication.

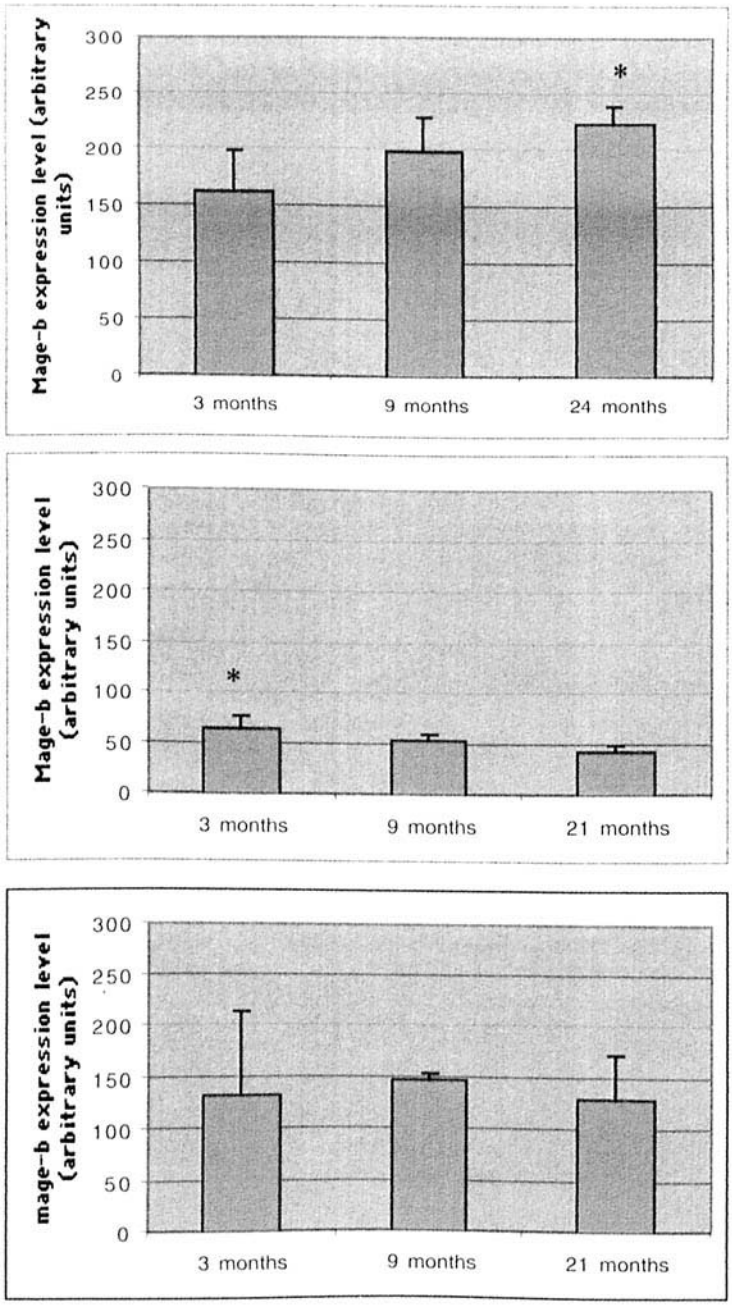

Figure 4. The effect of aging on the Mage-b expression levels in 64pT (A) or 4TO7cg primary breast tumors (B), or 4TO7cg metastases (C). Mage-b expression levels were determined by RT-PCR followed by Southern blotting. The intensity of the 1071 bp band was measured by a Gel Doc 2000. Each tumor was tested three times and the results were averaged per tumor. Subsequently, the Mage-b expression level per age group was averaged and subjected to statistical analysis using Tukey-Kramer multiple comparison test. Some tumors and all metastases were too small to repeat three times. The number of 64pT tumors analyzed was as follows: $n = 3$ for 3 months, $n = 6$ for 9 months, and $n = 6$ for 24 months. The number of 4TO7cg tumors analyzed was as follows: $n = 6$ for 3 months, $n = 5$ for 9 months, and $n = 14$ for 21 months. The number of 4TO7cg metastases analyzed was as follows: $n = 3$ for 3 and 9 months, and $n = 8$ for 21 months. Generally, each mouse developed one tumor at the place of injection. The 64pT tumors expressed significantly higher levels of Mage-b in mice of 24 months compared with mice of 3 months (*, $P < 0.05$), but not compared with mice of 9 months ($P > 0.05$). No significant difference was found in Mage-b expression levels when 64pT tumors were compared in mice from 9 versus 24 months ($P > 0.05$). The 4TO7cg tumors expressed significantly higher levels of Mage-b in mice of 3 months compared with mice of 21 months (**, $P < 0.001$), but not compared with mice of 9 months ($P > 0.05$). No significant difference was found in Mage-b expression levels when 4TO7cg tumors were compared in mice from 9 versus 21 months ($P > 0.05$). No significant difference was observed in Mage-b expression levels in 4TO7cg metastases between the different age groups ($P > 0.05$).

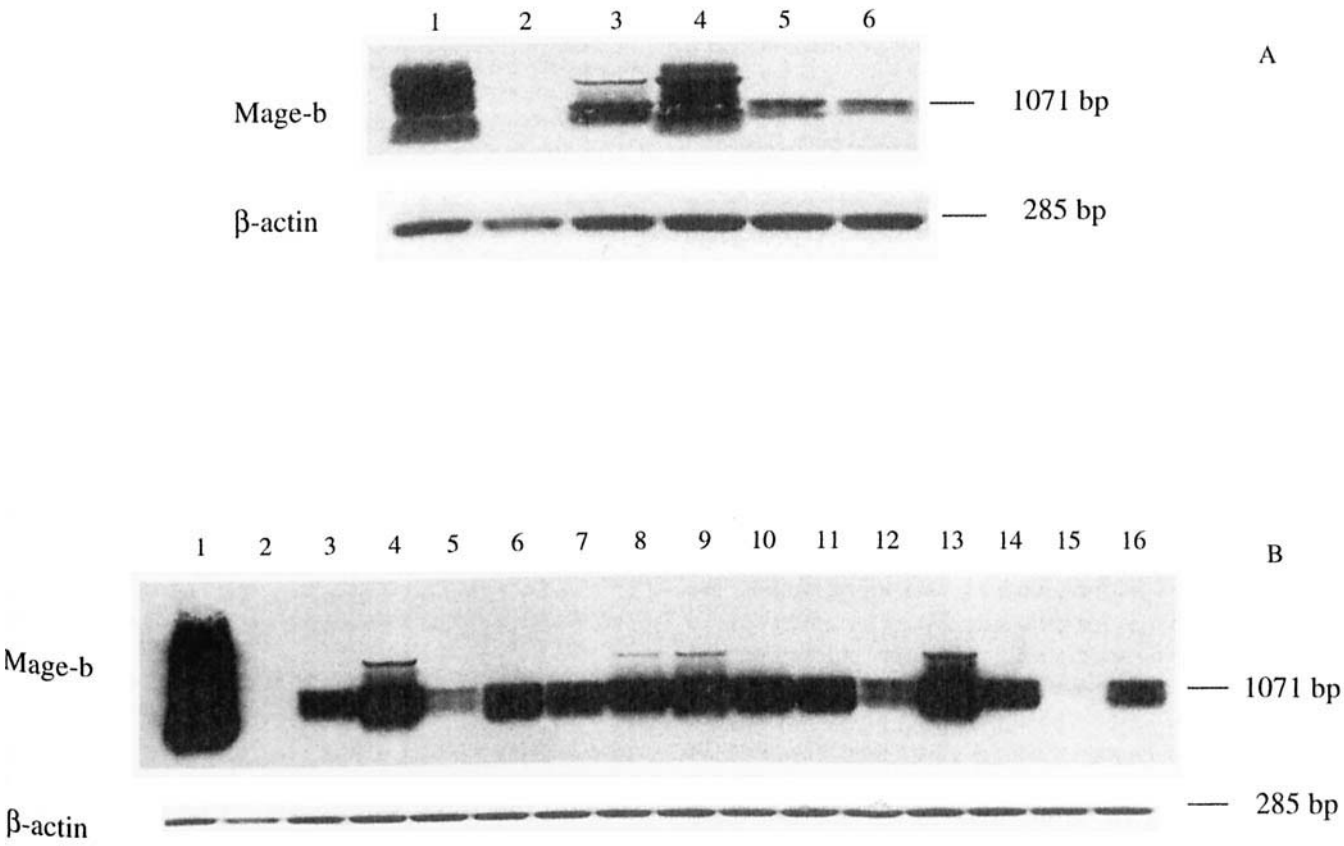

Figure 5. Representative example of Mage-b expression levels in 64pT and 4TO7cg primary breast tumors (A) and 4TO7cg metastases (B) at various ages. (Top) Southern blot hybridized with an Mage-specific probe. (Bottom) Ethidium bromide–stained agarose gel with RT-PCR products using β-actin primers. (A) Primary breast tumors: Lane 1, testis. Lane 2, normal breast tissue. Lane 3, 64pT tumor (3 months). Lane 4, 64pT tumor (24 months). Lane 5, 4TO7cg tumor (3 months). Lane 6, 4TO7cg tumor (21 months). (B) Metastases: Lane 1, testis. Lane 2, normal breast tissue. Lane 3, peritoneal cavity (3 months). Lane 4, peritoneal cavity (3 months). Lane 5, diaphragm (3 months). Lane 6, peritoneal cavity (9 months). Lane 7, liver (9 months). Lane 8, lung (9 months). Lane 9, peritoneal cavity (9 months). Lane 10, diaphragm (9 months). Lane 11, peritoneal cavity (21 months). Lane 12, lymph node (21 months). Lane 13, peritoneal cavity (21 months). Lane 14, peritoneal cavity (21 months). Lane 15, lung (21 months) and peritoneal cavity (21 months).

tumor progression observed. We found a significant decrease in the percentage of $CD4^+$ T lymphocytes in the inguinal lymph nodes of old as compared with young mice, most prominently in mice without tumors, but a significant decrease was also found in the ones with 4TO7cg tumors, and to a lesser extent (not significant) in the ones with 64pT tumors. This suggests that the decrease in the percentage of $CD4^+$ T lymphocytes is age-related but not tumor-specific and would be in keeping with a possible increased T-cell unresponsiveness. Others found a continuous decline in the proportion of $CD4^+$ T lymphocytes in the peripheral blood of BALB/c and C57B6 mice as a function of age (37).

In contrast to the $CD4^+$ T lymphocytes, we found a significant increase in the percentage of $CD8^+$ T lymphocytes in inguinal lymph nodes of old mice with 64pT tumors as compared with their young counterparts. However, in the mice without tumors, a slight decrease in the percentage of $CD8^+$ T cells was observed. Surprisingly, this suggests that $CD8^+$ T cells were attracted by the tumors in old mice, whereas the general consensus is that T cells are less responsive at old age. A similar trend was observed in the 4TO7cg mice, albeit this was not statistically significant. The observed increase in $CD8^+$ T lymphocytes was associated with a significant increase in Mage-b expression levels in the 64pT primary breast tumors at old age. Whether these higher Mage-b expression levels attracted $CD8^+$ T cells to the draining lymph nodes and contributed to the smaller tumors in the older animals is as yet unclear.

If older mice still can attract $CD8^+$ T cells to the tumor, and generally have a less permissive microenvironment for tumor growth, what causes the increased tumor incidence and mortality at old age? In this respect, it should be realized that our present study only models the later progressive stages of a tumor. Initiating factors, such as increased genomic instability (38) and increased tissue disorganization (39) are likely to play an important role, as do other factors specific for old age, such as increased frailty.

What we specifically addressed in this study was the question of whether our mouse models are useful to develop and optimize a relatively benign, yet effective, alternative to current drug or radiation-based breast cancer therapies. Such a treatment—one that would specifically reduce or eliminate

distant metastases or residual tumor cells—offers great promise in the outcome. Our observations indicate neither a significant reduction in $CD8^+$ T cell responses nor a reduced concentration of tumor-associated antigens, two key factors for a vaccine strategy to succeed. Since MAGE has been frequently detected in primary breast tumors and metastases (21, 40), a MAGE-encoding vaccine would be of great value in breast cancer therapies. Therefore, we compared Mage expression levels in metastases and primary tumor in our mouse 4TO7cg model with MAGE expression levels in humans. We found high expression levels of Mage-b in almost all metastases, regardless of age. The expression levels were 2- to 3-fold higher in the metastases than in the primary 4TO7cg breast tumors. In humans, MAGE has also been more frequently detected in metastases of metastatic melanoma (48%) than in primary tumors (16%; Ref. 41).

Our finding of the high Mage-b expression levels in the 4TO7cg metastases, in combination with the attraction of $CD8^+$ T cells to the tumors in old mice, fulfills two important criteria for the success of a breast cancer vaccination approach specific for the elderly patient. However, it should be realized that it is difficult to extrapolate the results obtained with our mouse models directly to the human situation. Most human tumors are weakly immunogenic, and for this reason it is essential to select optimal TAA as the basis for an effective breast cancer vaccine. The mouse models used in this study are highly suitable to optimize experimental cancer vaccines based on mouse homologues of the human MAGE TAA. Indeed, preliminary results obtained with a Mage-b DNA vaccine indicate the induction of Mage-b–specific immune responses and a protective effect against 4TO7cg metastases in young BALB/c mice.

1. Reichert JM, Paquette C. Therapeutic cancer vaccines on trial. Nature Biotechnol 20:659–663, 2002.
2. Muss HB. Factors used to select adjuvant therapy of breast cancer in the United States: an overview of age, race, and socioeconomic status. J Natl Cancer Inst Monogr 30:52–55, 2001.
3. Kurtz JE, Dufour P. Strategies for improving quality of life in older patients with metastatic breast cancer. Drugs Aging 19:605–622, 2002.
4. Shah S, Doyle K, Lange EM, Shen P, Pennell T, Ferree C, Levine EA, Perrier ND. Breast cancer recurrence in elderly patients after lumpectomy. Am Surg 68:735–739, 2002.
5. Wiegel T, Kreusel KM, Bornfeld N, Bottke D, Stange M, Foerster M, Hinkelbein W. Frequency of asymptomatic choroidal metastasis in patients with disseminated breast cancer: results of a prospective screening study. Br J Opthalmol 82:1159–1161, 1998.
6. Albergh AJ, Sing S. Epidemiology of breast cancer in older women: implications for further health care. Drugs Aging 18:761–762, 2001.
7. Ershler WB. The aggressiveness of neoplasms with age. Geriatrics 42:99–103, 1984.
8. Tsuda T, Kim YT, Siskind GW. Role of the thymus and T cells in slow growth of B16 melanoma in old mice. Cancer Res 47:3097–3102, 1987.
9. Rockwell S. Effect of host age on the transplantation, growth, and radiation response of EMT6 tumors. Cancer Res 41:527–531, 1981.
10. Itzhaki O, Skutelsky E, Kaptzan T, Siegal A, Michowitz M, Sinai J, Huszar M, Nafar S, Leibovici J. Macrophage-recognized molecules of apoptotic cells are expressed at higher levels in AKR lymphoma of aged as compared to young mice. Adv Exp Med Biol 479:251–262, 2000.
11. Miller RA. The aging immune system: primer and prospectus. Science 273:70–74, 1996.
12. Molls RR, Ahluwalia N, Fick T, Mastro AM, Wagstaff D, Handte G, Ball R. Inter- and intra-individual variation in tests of cell mediated immunity in young and old women. Mech Aging Dev 124:619–627, 2003.
13. George AJT, Ritter MA. Thymic involution with aging: obsolescence or good housekeeping? Immunol Today 17:267–272, 1996.
14. Patriarcia PA. A randomized controlled trial of influenza vaccine in the elderly. Scientific scrutiny and ethical responsibility. JAMA 272:1700–1701, 1994.
15. McElhaney JE, Meneilly GS, Lechelt KE, Bleackley RC. Split-virus influenza vaccines: do they provide adequate immunity in the elderly? Gerontology 49:M37–M43, 1994.
16. Gravekamp C, Bontenbal M, Ronteltap CPM, van Duivenbode D, Bolhuis RLH. *In vitro* and *in vivo* activation of CD4+ T lymphocytes by autologous tumor cells. Int J Cancer 46:152–154, 1990.
17. Schreiber H. Tumor immunology. In: Paul WE, Ed. Fundamental Immunology (4th ed.). Philadelphia, PA: Lippincott-Raven, pp1237–1270, 1998.
18. Koyama S, Maruyama T, Adachi S, Nozue M. Expression of co-stimulatory molecules, B7-1 and B7-2 on human gastric carcinoma. J Cancer Res Clin Oncol 124:383–388, 1998.
19. Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumors. Immunol Today 18:89–95, 1997.
20. Akbasak A, Oldfield EH, Saris SC. Expression and modulation of major histocompatibility antigens on murine brain tumors *in vitro*. J Neurosurg 75:922–929, 1991.
21. Park JW, Kwon TK, Kim IH, Sohn SS, Kim CI, Bae OS, Lee KS, Lee KD, Lee CS, Chang HK, Choe BK, Ahn SY, Jeon CH. A new strategy for the diagnosis of MAGE-expressing cancers. J Immunol 79:79–86, 2002.
22. Rosenberg SA, Zhai Y, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian, SL, Restifo NP, Seipp CA, Einhorn JH, Roberts B, White DE. Immunizing patients with metastatic melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. J Natl Cancer Inst 90:1984–1900, 1998.
23. Anichini A, Molla A, Mortarini R, Tragni G, Bersani I, Nicola MD, Gianni M, Dunbar R, Cerundolo V, Parmiani G. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J Exp Med 190: 651–668, 1999.
24. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, van Wijck R, Lienaard D, Beauduin M, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 80:219–230, 1999.
25. De Backer O, Verheyden AM, Martin B, Godelaine D, De Plaen E, Brasseur R, Avner P, Boon T. Structure, chromosomal location, and expression pattern of three mouse genes homologous to the human MAGE genes. Genomics 28:74–83, 1995.
26. De Plaen E, De Backer O, Arnaud D, Bonjean B, Chomez P, Martelange V, Avner P, Baldacci P, Babinet C, Hwang SY, Knowles B, Boon T. A new family of mouse genes homologous to the human MAGE genes. Genomics 55:176–184, 1999.
27. Sypniewska RK, Hoflack L, Bearss DJ, Gravekamp C. Potential mouse

tumor model for pre-clinical testing of Mage-specific breast cancer vaccines. Breast Cancer Res Treat 74:221–233, 2002.

28. Sypniewska RK, Hoflack L, Tarango M, Gauntt S, Reddick RL, Gravekamp C. Prevention of metastases with a Mage-b DNA vaccine in a mouse breast tumor model: potential for breast cancer therapy. Breast Cancer Res Treat (submitted).
29. Guayerbas N, De la Fuente M. An impairment of phagocytic function is linked to a shorter life span in two strains of premature mice. Dev Comp Immunol 27:339–350, 2003.
30. Rak JW, McEachern D, Miller FR. Sequential alteration of peanut agglutinin binding-glycoprotein expression during progression in murine mammary neoplasia. Br J Cancer 65:641–648, 1992.
31. Aslakson CJ, McEachern D, Conaway DH, Miller FR. Inhibition of lung colonization at two different steps in the metastatic sequence. Clin Exp Metastasis 9:139–150, 1991.
32. Miller FR, Aslakson CJ, McEachern D. Modulation of host resistance to metastasis in the lungs of aged retired breeder mice. Invasion Metastasis 11:233–240, 1991.
33. Reeves JP, Reeves PA. Care and handling of laboratory animals. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, Eds. Current Protocols in Immunology. New York: Wiley, Vol 1:pp1.9.1, 2003.
34. Gravekamp C. Tailoring cancer vaccines to the elderly: the importance of suitable mouse tumor models. Mech Aging Dev 122:1087–1105, 2001.
35. Yancik R, Ries LG, Yates JW. Breast cancer in aging women. A population-based study of contrasts in stage, surgery, and survival. Cancer 63:976–981, 1989.
36. Bronson RT. Rate of occurrence of lesions in 20 inbred and hybrid genotypes of rats and mice sacrificed at 6 month intervals during the first year of life. In: Harrison DE, Ed. Genetics of Aging II. Caldwell, NJ: Telford Press, pp 280–358, 1990.
37. Chen J, Flurkey K, Harrison DE. A reduced peripheral blood CD4(+) lymphocyte proportion is a consistent aging phenotype. Mech Aging Dev 123:145–153, 2002.
38. Vijg J. Somatic mutations and aging: a re-evaluation. Mutat Res 447:117–135, 2000.
39. Campisi J. Cancer and ageing: rival demons? Natl Rev Cancer. 3:339–349, 2003.
40. Wascher RA, Bostick PJ, Huynh KT, Turner R, Qi K, Giuliano AE, Hoon DS. Detection of MAGE-A3 in breast cancer patients' sentinel lymph nodes. Br J Cancer 85:1340–1346, 2001.
41. Brasseur F, Rimoldi D, Lienard D, Lethe B, Carrel S, Arienti F, Suter L, Vanwijck R, Bourlond A, Humblet Y. Expression of MAGE genes in primary and metastatic cutaneous melanoma. Int J Cancer 63:375–380, 1995.