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The elucidation of the immune response to cancer should be of great help in the development of new therapeutic strategies for the treatment of breast cancer. Based on recent advances in our basic understanding of antigen recognition by T lymphocytes, it has been possible to identify several human tumor-associated antigens (TAA) recognized by CTLs. However, the expression for these TAAs has been shown to be relatively low in BC tumor cells. A new protein named mammaglobin has been demonstrated to be exclusively expressed in the mammary epithelium. In addition, 90% of primary BC tumors have detectable levels of mammaglobin protein Given the exclusive mammaglobin expression in BC tumors, this novel protein may prove to be a TAA highly specific for BC that could be utilized in the near future for *in vitro* BC-specific activation of CTLs. The discovery of mammaglobin-derived antigenic peptides that are highly expressed in BC tumor tissue and are recognized by CTLs offer many exciting future therapeutic options for the treatment of BC.

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INTRODUCTION

A novel breast cancer-specific protein, named mammaglobin, has been identified in our laboratories. Analyses of 15 human adult and 3 fetal tissues have demonstrated that mammaglobin is exclusively expressed in the mammary epithelium during proliferation and terminal differentiation. Significantly, high expression of mammaglobin has been found in 50% of human breast cancer cell lines as well as in 62% metastatic primary breast cancer tumors. Therefore, the characterization of the immune response to a specific and highly expressed breast cancer-associated protein, such as mammoglobin should be of great importance toward the development of new immuno-therapeutic strategies for the treatment and prevention of this disease.

BODY OF WORK

Task 1. To determine whether breast cancer-specific T cell immune responses generated *in vivo* can recognize mammaglobin-derived antigenic peptides.

1. Lack of mammaglobin-specific T cell proliferative responses in healthy female individuals as compared to healthy male individuals. The expression of mammaglobin has been shown to be restricted to the normal adult mammary epithelium and is overexpressed in 23% of primary breast tumors. Therefore, normal individuals would show specific T cell unresponsiveness (tolerance) against mammaglobin. However, the possibility exists that the tissue-specific mammaglobin expression may be regulated by steroid hormones as is the expression of all the uteroglobin family member. This hormone-regulated expression of mammaglobin may lead to a differential expression of mammaglobin in the adult mammary epithelium between healthy female and male individuals and hence to differential levels of T cell responsiveness to this same autoantigen. To test this, we produced recombinant mammaglobin by subcloning the mammaglobin cDNA into a PCI-neo expression vector and consequent transformation of E. coli. Then, we compared the T cell proliferative responses to recombinant mammagloblin between healthy adult female and male individuals as described (See attached manuscript, Appendix 1). As observed in Figure 1 (Appendix 1), peripheral blood mononuclear cells (PBMC) derived from healthy adult male individuals showed significantly higher levels of proliferation to mammaglobin as compared to PBMCs from healthy female individuals. Interestingly, no proliferative responses were observed in healthy adult males or females individuals to another autoantigen, human albumin, which is expressed in comparable levels in individuals from both sexes. These data indicate that adult female individuals are tolerant to mammaglobin. Therefore, mammaglobin-specific T cells in healthy adult females may be absent from the periphery (clonal deletion) or display antigen-specific unresponsiveness against mammaglobin (anergy).

2. Higher frequency of mammaglobin-reactive T cells in breast cancer patients than in healthy female individuals. To address the question whether mammaglobin-specific T cells are deleted or anergized in adult females, we determined the precursor frequency of mammaglobin reactive T cells in healthy adult female individuals (controls) and breast cancer patients. Briefly, we determined the frequency of mammaglobin-reactive CD4+ T helper cells and CD8+ cytotoxic T cells using autologous dendritic cells pre-pulsed with recombinant mammaglobin (See attached manuscript. Appendix 1). If the mammaglobin-specific T cells are anergic, the possibility exists that selective mammaglobin-specific tolerant T cells clones may respond to this protein in case of aberrantly high expression of the protein as observed in breast tumors. In this case, we would observe a higher frequency of mammaglobin-reactive T cells in a selected group of breast cancer patients. On the other hand, if mammaglobin-specific T cell clones had been deleted we would not be able to observe a higher frequency of mammaglobin-reactive T cells in breast cancer patients. As shown in Table 2 (Appendix 1), the frequency of mammaglobin-specific proliferating T cells in peripheral blood of breast

cancer patient ($3.5x10^{-5} \pm 3.2x10^{-5}$) were significantly higher than that observed in healthy female control individuals ($0.15x10^{-5} \pm 0.17x10^{-6}$) (P=0.03, Student's T tests). Only one of the seven patients included in this study did not respond to recombinant mammaglobin (patient No. 7).

In addition, the frequency of mammaglobin-reactive CD8+ CTLs in peripheral blood of breast cancer patients ($2.4x10^{-5} \pm 1.8x10^{-5}$) was also significantly higher than that observed in healthy female control individuals ($0.39x10^{-5} \pm 0.52x10^{-5}$) (P=0.03, Student's T test) (Table 2, Appendix 1). Interestingly, one of the seven healthy female control individuals included in this study showed a particularly high CTL precursor frequency (control No. 4).

The results presented herein indicate that, even though healthy female individuals do not respond to mammaglobin as a normal auto-antigen, they have mammaglobin-specific T cell clones that expand *in vivo* as result of an aberrant over-expression of this protein in breast cancer cells.

Task 2. To determine whether mammaglobin-reactive CD8+ CTLs generated *in vitro* have the ability to lyse breast cancer cells.

1. Mammaglobin-specific proliferative response by CD8+ CTL lines generated *in vitro*. Based on the observation that mammaglobin-specific CTLs are normally expanded in breast cancer patients *in vivo*, the possibility existed that high affinity mammaglobin-specific CTL lines could be generated *in vitro*. To test this, we generated a mammaglobin-specific CD8+ T cell line from a HLA-A2-positive, breast cancer patient by means of five weekly stimulations with dendritic cells pre-pulsed with recombinant mammaglobin or two different HLA-A2-binding, mammaglobin-derived peptides (SKM-67: KLLMVLMLA [aminoacids 2-10] and SKM-68: LLMVLMLAA [aminoacids 3-11]) (See attached manuscript, Appendix 1). The HLA-A2-binding capabilities of these mammaglobin-derived peptides were predicted by means of the Bioinformatics and Molecular Analysis Section Program (www.bimas.dcrt.nih.gov/molbio/hla_bind/). The SKM-67 and SKM-68 peptides were used in these studies because they presented the highest affinities (longer complex disassociation times) for the HLA-A2 molecule. As shown in Figure 2 (Appendix 1), the resulting CD8+ CTL line produced against recombinant mammaglobin showed a significantly high proliferative response in the presence of recombinant mammaglobin but not in the presence of bovine serum albumin (control 1) or human albumin (control 2). This data indicate the mammaglobin-specificity of this reaction.

In parallel experiments, the peptide-specific proliferation of the CD8+ CTL lines generated against the SKM-67 and SKM-68 peptides was also determined. As shown in Figures 3A and 3B (Appendix 1), both of these CD8+ CTL lines demonstrated peptide-specific proliferation upon stimulation with cognate peptide, respectively. Since both peptides shared the common anchor residues (L in position 2 and A in position 9), proliferation was observed in reciprocal combination, although the responses were weaker as compared to the corresponding cognate peptide.

2. Mammaglobin-specific cytotoxic activity by CD8+ T cell lines generated *in vitro*. The results shown above indicate the specific reactivity against mammaglobin of three different CD8+ CTL lines generated *in vitro*. To analyze the mammaglobin-specific CTL activity of the CD8+ T cell line generated against recombinant mammaglobin, we determined its ability to lyse autologous dendritic cells pulsed with recombinant mammaglobin. As shown in Figure 4 (Appendix 1), this particular CD8+ CTL line demonstrated antigen-specific killing of recombinant mammaglobin-pulsed autologous dendritic cells. No lytic activity was observed with non-pulsed autologous dendritic cells (control 1) as well as autologous dendritic cells pulsed with human albumin (control 2). The lytic activity observed with the mammaglobin-pulsed dendritic cells was significantly inhibited by pre-treatment with the W6/32 anti-HLA-class I monoclonal antibody but not with the KUIA-2 anti-HLA-DR monoclonal antibody. These data demonstrate the HLA class I-restricted recognition of mammaglobin-derived epitopes by this CD8+ CTL line.

In addition, as shown in Figure 5A and 5B (Appendix 1), CD8+ CTL lines obtained in vitro against the

SKM-67 and SKM-68 peptides also show a significant CTL activity against T2 cells pulsed with the corresponding cognate peptide. This result indicates that both SKM-67 and SKM-68 are recognized in the context of HLA-A2 the CTL lines generated *in vitro*.

3. Mammaglobin-specific MHC-restricted cytotoxic activity on breast cancer cells by CD8+ T cells generated *in vitro*. The CD8+ CTL line generated in vitro against autologous dendritic cells pre-pulsed with recombinant mammaglobin was tested for its ability to lyse several breast cancer cell lines that naturally express the mammaglobin protein (Table 1, attached manuscript). As shown in Figure 6 (Appendix 1), this CD8+ CTL line selectively killed mammaglobin-positive breast cancer cell lines in the context of HLA-A2 (HBL-100 and AU565). No killing was observed with mammaglobin-positive, HLA-A2-negative breast cancer cell lines (MDA-MB-415 and MDA-MB-361) or mammaglobin-negative, HLA-A2-positive breast cancer cell lines (MCF-7 and MDA-MB-231). These results clearly demonstrate the MHC restriction and antigen specificity of the CTL activity mediated by mammaglobin-specific CD8+ CTLs generated *in vitro*.

Task 3. To determine the mammaglobin CD8+ T cells epitopes presented by HLA-1, HLA-A2 and HLA-A3.

1. Mammaglobin-specific, HLA-A3-restricted, cytotoxic activity by CD8+ T cells generated *in vitro* is directed against one dominant antigenic peptide. In order to identify the dominant mammaglobin-derived epitope(s) presented in the context of the HLA-A3 molecule, we developed two CD8+ T cell lines from one HLA-A3 individual by means of culture in the presence of HLA-A3-transfected T2 cells (T2-HLA-A3+) pre-pulsed with eight HLA-A3-binding, mammaglobin-derived peptides (MG-2: FLNQTDETL, MG-3: LMVLMLAAL, MG-5: KLLMVLMLA, MG-6: TTNAIDELK, MG-7: KTINPQVSK, MG-9: PLLENVISK, MG-11: LMLAALSQH, and MG-12: AIDELKECF). The prediction of these epitopes was made using the Bioinformatics and Molecular Analysis Section program (www.bimas.dcrt.nih.gov/molbio/hla_bind/). These peptides were used in these studies because they presented the highest affinities for the HLA-A3 molecule. Briefly, After monocyte depletion, peripheral blood mononuclear cells were cultured in presence of irradiated, peptide-loaded T2-HLA-A3+ cells in the presence of recombinant IL-2. After five weekly stimulations, a standard ^{51}Cr release assay was performed using T2-HLA-A3+ cells pulsed with the mammaglobin-derived peptides as targets. As shown in Figure 1 (Appendix 2), significant cytotoxic activity was observe only against the T2-HLA-A3+ cells loaded with the peptide PLLENVISK (MG-9, aminoacids 23-31). No significant killing was observed against T2-HLA-A3 cells loaded with the other mammagloblin-derived peptides. These results clearly indicate that even though this CD8+ T cell line was developed against several HLA-A3-binding peptides, only the MG-9 peptide is able to stimulate the development of CD8+ CTLs. Similar experiments are currently being performed in our laboratory to determine the dominant mammaglobin epitopes presented in the context of HLA-A2 molecules.

In order to determine whether the CD8+ CTL line directed against the MG-9 peptide had the ability to lyse HLA-A3-positive breast cancer cells that express mammaglobin, we performed a CTL activity assay using as targets several breast cancer cell lines (Table 1, Appendix 2). As shown in Figure 2 (Appendix 2), the anti-MG-9 CTL line showed significant cytotoxic activity against one mammaglobin-positive, HLA-A3-positive breast cancer cell line (DU4475). No killing was observed in K-562 cells which indicates the lack of NK cell activity. No CTL activity was observed against mammaglobin-negative, HLA-A3-positive breast cancer cells (T-47D), mammaglobin-positive, HLA-A3-negative breast cancer cells (HBL-100), and mammaglobin-negative, HLA-A3-negative breast cancer cells (MCF-7).

The results presented herein clearly indicate that anti-mammaglobin CD8+ T cell lines developed *in vitro* have the ability to kill breast cancer cells in a MHC-restricted manner and that the MG-9 peptide is a naturally produced mammaglobin epitope presented by HLA-A3 breast cancer cells. Interestingly, no CTL

activity was observed against one mammaglobin-positive, HLA-A3-positive breast cancer cell line (AU-565). However, flow cytometric analysis performed to determine the levels of expresssion of the HLA-A3 gene in this cell line showed no HLA-A3 expression as compared to the DU4475 cell line. This result further indicate that the MG-9 peptide is naturally expressed in the context of the HLA-A3 molecule.

2. Epitope immunodominance of the anti-mammaglobin CD8+ CTL response developed *in vivo* in breat cancer patients. As shown above, we observed that breast cancer patients developed a detectable CD8+ CTL response to mammaglobin *in vivo* (Table 1, Appendix 1). To identify the mammaglobin-derived determinant(s) recognized by CD8+ CTLs in breast cancer patients, we have performed anti-IFN-gamma ELISPOT assays in five HLA-A3 breast cancer patients and five HLA-A2 breast cancer patients. Toward this, we have stimulated PBMCs from these patients with the eight HLA-A3-binding, mammaglobin-derived peptides (see above) or seven HLA-A2-binding, mammaglobin derived peptides (MG-1: TLSNVEVFM, MG-2: FLNQTDETL, MG-3: LMVLMLAAL, MG-4: TINPQVSKT, MG-5: KLLMVLMLA, MG-8: LIYDSSLCDL, and MG-10: MQLIYDSSL). As mentioned above, the prediction of the HLA-A2 epitopes was made using the Bioinformatics and Molecular Analysis Section program (www.bimas.dcrt.nih.gov/molbio/hla_bind/). These peptides were used in these studies because they presented the highest affinities for the HLA-A2 molecule. Briefly, $2x10^5$ PBMCs were cultured in the presence of each individual peptide in 96-well plates. After 48 hours, the frequency of IFN-gamma-producing cells was determined by means of a standard ELISPOT assay. As observed on Figure 3 (Appendix 2), results with the HLA-A2 breast cancer patients show that they have a significant reactivity against the peptides FLNQTDETL (MG-2, aminoacids 66-74) and LIYDSSLCDL (MG-8, aminoacids 83-92). In addition, as observed in Figure 4 (Appendix 2), results with the HLA-A3 breast cancer patients show that they have a significant reactivity to the peptides KLLMVLMLA (MG-5, aminoacids 2-10) and TTNAIDELK (MG-6, aminoacids 55-63). Additional ELISPOT assays are currently being performed in our laboratory to expand the number of patients and peptides in these analyses.

The results presented herein clearly identify the dominant mammaglobin-derived epitopes recognized in the context of HLA-A2 and HLA-A3 molecules by CD8+ CTLs *in vivo*. Future experiments will be performed to determine whether HLA-A2 and HLA-A3 CD8+ CTL lines developed *in vitro* against these particular peptides will have the ability to lyse HLA-matched, mammaglobin-positive breast cancer cells *in vitro*. It is interesting that the HLA-A3 CD8+ CTL line generated *in vitro* against the pool of mammaglobin-derived peptides presented a different epitope dominance (Figure 1, Appendix 2) as compared to the dominance observed *in vivo* (Figure 4, Appendix 2). This difference in the results may be explained by the fact that the antigen presenting cells in our in vitro experiments were T2-HLA-A3+ cells and not natural antigen presenting cells such as macrophages and dendritic cells. It remains possible that peptide determinants that are cryptic when presented by one type of antigen presenting cell become immunodominant when presented by other. However, it is noteworthy that the anti-mammaglobin CD8+ CTL line generated *in vitro* showed a highly significant cytotoxic activity against breast cancer cells (Figure 2, Appendix 2).

<u>Task 4. To determine the potential use of mammaglobin cDNA vaccination in the generation of breast cancer-specific, HLA-restricted CTL immune response.</u>

We have injected HLA-A2 mice intramuscularly with 100 μg of mammaglobin cDNA in the PCI-neo expression vector and the necrotizing agent Marcaine. The DNA construct was injected every two weeks for a total of three inoculations. Cytotoxic activity against mammaglobin-positive, HLA-A2-positive human breast cancer cells (Table 1, Appendix 2) was not detected as compared to non-injected control mice. In addition, no proliferative responses were detected in these animals against recombinant mammagloblin as compared to the response observed in non-injected control mice. In addition, no anti-mammaglobin

antibodies were detected in the injected mice.

Based on these negative results we plan to repeat the DNA immunization protocol with an increased dose of mammaglobin DNA (200 μg). In addition, it has been reported that mouse CD8 does not interact optimally with human MHC molecules. For this reason we have obtained human CD8 (hCD8) transgenic mice and we will perform the experiments proposed in double transgenic HLA-A2 and hCD8 mice where the interaction between CD8 and MHC will be optimal for *in vivo* as well as *in vitro* (cytotoxic activity assays with human breast cancer cells) interactions.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that breast cancer patients have higher frequency of mammaglobin-reactive proliferating CD4+ T cells as well as CD8+ CTLs in peripheral blood as compared to normal female individuals.

- We have identified that the mammaglobin epitopes recognized by CD8+ CTLs *in vivo* in the context of the HLA-A2 molecule are the peptides FLNQTDETL (aminoacids 66-74) and LIYDSSLCDL (aminoacids 83-92).

- We have identified that the mammaglobin epitopes recognized by CD8+ CTLs *in vivo* in the context of the HLA-A3 molecule are the peptides KLLMVLMLA (aminoacids 2-10) and TTNAIDELK (aminoacids 55-63).

- We have demonstrated that anti-mammaglobin CD8+ T cell lines developed in vitro by means of different protocols display MHC-restricted, mammaglobin-specific cytotoxic activity against breast cancer cells.

- We have identified that the mammaglobin epitope recognized by CD8+ CTLs generated *in vitro* in the context of the HLA-A3 molecule is the peptide PLLENVISK (aminoacids 23-31).

REPORTABLE OUTCOMES

Two peer-reviewed manuscripts have been supported by this award:

1. **Manna PP, Jaramillo A, Majumder K, Fleming TP, Doherty G, Dipersio JF, Mohanakumar T.** 2001. Mammaglobin, a tumor-associated antigen recognized on human breast cancer cells by cytotoxic T lymphocytes. Journal of the National Cancer Institute: Submitted (Appendix 1).

2. **Majumder K, Jaramillo A, Fleming TP, Doherty G, Dipersio JF, Mohanakumar T.** 2001. Definition of a mammaglobin-derived epitope recognized by cytotoxic T lymphocytes on breast cancer cells. In preparation (Appendix 2).

CONCLUSIONS

Breast cancer patients developed both CD4+ and CD8+ T cell immune responses to mammaglobin *in vivo*. In addition, mammaglobin-specific CD8+ T cell lines generated in vitro show significant cytotoxic activity against breast cancer cells *in vitro*. These results indicate that mammaglobin is a tumor-associated antigen recognized by CD8+ T cells and could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

REFERENCES

None used.

Mammaglobin, a Tumor-Associated Antigen Recognized on Human Breast Cancer Cells by Cytotoxic T Lymphocytes

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Abbreviations: BSA, bovine serum albumin; CPM, counts per minute; CTL, cytotoxic T lymphocyte; HA, human albumin; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell.

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Abstract

The mammaglobin gene is localized in chromosome 11q13 and encodes a 10 kDa glycoprotein that is highly over-expressed in human breast tumor cell lines and primary breast tumors. Since the pattern of expression of mammaglobin is restricted to mammary epithelium and metastatic breast tumors, the induction of T cell-mediated immune responses is immensely important for designing specific tumor-targeted immunotherapy. In this study, we investigated whether mammaglobin-specific T cells are present in healthy individuals as well as in breast cancer patients and the feasibility to develop mammaglobin-specific cytotoxic T lymphocytes (CTL) *in vitro* with the capacity to lyse breast cancer cells in a major histocompatibility antigen-restricted manner. We show herein that T cells from healthy adult male individuals display significantly higher levels of proliferation to mammaglobin as compared to healthy female individuals. However, the frequency of mammaglobin-specific proliferating T cells as well as CTLs in breast cancer patient was significantly higher than that observed in healthy female individuals (P=0.03, two-tailed student's T test). In addition, mammaglobin-specific CD8+ T cell lines developed *in vitro* from an HLA-A2-positive breast cancer patient against recombinant mammaglobin or mammaglobin-derived peptides showed significantly high proliferative responses in the presence of recombinant mammaglobin or the corresponding peptide, respectively. These CD8+ T cell lines also demonstrated significant mammaglobin-specific CTL activity. One of these CD8+ T cell lines developed against recombinant mammaglobin displayed antigen-specific, major histocompatibility antigen-restricted CTL activity against breast cancer cells *in vitro*. These results suggest that mammaglobin could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

Introduction

The evolution of breast cancer is accompanied by multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). To date most of the studies have concentrated on the MAGE and Erb-b2 families of tumor-associated genes in a variety of cancers (2-6). With the exception of the Her-2/neu and BRCA-1/2 oncogenes, there is only limited information available regarding the involvement of other genes in breast cancer (7,8). The mammaglobin gene was first identified using a differential screening approach directed at the isolation of novel human breast cancer-associated gene (9,10). The human mammaglobin gene is localized in chromosome 11q13 and encodes a 10 kDa glycoprotein that is distantly related to a family of epithelial secretory proteins that includes rat estramustine-binding protein/prostatein and human Clara cell protein (CC10/uteroglobulin) (11-14).

Mammaglobin demonstrates several properties as a clinically relevant breast cancer-associated marker. Unlike Erb/b (15) or cyclin (16), two other genes that are over-expressed in breast cancer, the over-expression of mammaglobin seems to be breast cancer-specific. The over-expression of this gene may reflect a more cell specific alteration of the mammary epithelium rather than representing a general increased growth potential or mitotic rate (10). Mammaglobin is expressed in early non-invasive ductal carcinoma as well as in late stage invasive disease. Although the function of the mammaglobin protein is unknown, it displays two characteristics to suggest that its expression is particularly relevant to breast cancer biology: northern blot as well as reverse transcriptase-polymerase chain reaction analyses of several adult human tissues have demonstrated that mammaglobin expression is restricted to the mammary gland and is highly overexpressed in human breast tumor cell lines and primary breast tumors (10). In addition, several studies have shown that mammaglobin mRNA is highly up-regulated in peripheral blood

of breast cancer patients (17-19). Furthermore, previous studies have reported that lymph nodes from patients with metastatic breast cancer contain detectable mammaglobin mRNA whereas in uninvolved nodes the expression was not detected (20-22).

Since the pattern of expression of mammaglobin is largely restricted to mammary epithelium and metastatic breast tumors, induction of primary cell-mediated immune response is immensely important for designing tumor targeted immunotherapy. In this communication, we show that breast cancer patients have significantly higher frequency of mammaglobin responsive proliferating as well as cytotoxic T lymphocytes (CTL) as compare to normal individuals. We also show the induction of a major histocompatibility antigen (MHC)-restricted CTL response against mammaglobin from peripheral blood of breast cancer patients.

Materials and Methods

Cell lines. A list of the breast cancer cell lines used in this study is given in Table 1. All breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured following the distributor's instructions. HLA typing of breast cancer cell lines and breast cancer patients was performed by oligoneucleotide sequence-specific primers that provided low to medium resolution for HLA-A genes (PEL-FREEZ, Brown Deer, WI) and high resolution for HLA-A2 genes (GenoVision Inc., Exton, PA) according to the manufacture's instruction. Mammaglobin expression was determined by reverse transcriptase-polymerase chain reaction using sequence-specific primers as previously described (10).

Proteins and peptides. Recombinant human mammaglobin was produced with the PCI-neo expression vector (Promega, Madison, WI) containing the full-length mammaglobin cDNA. Human albumin (HA) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO). Two HLA-A2-binding mammaglobin-derived peptides, SKM-67 (KLLMVLMLA) and SKM-68 (LLMVLMLAA) were used in this study. An HLA-A3-binding peptide, FK-31 (GPPGVTIVK), was used as irrelevant control peptide in this study (24). Peptides were synthesized using an Applied Biosystems Synergy peptide synthesizer (Model 432A, Foster City, CA). The purity of peptides was greater than 95% as evidenced by high-performance liquid chromatography and mass spectrometry. The peptides were dissolved in sterile distilled water at a stock concentration of 10 mg/ml before use. The HLA-A2-binding mammaglobin-derived peptides were predicted by means of the Bioinformatics and Molecular Analysis Section program (www.bimas.dcrt.nih.gov/molbio/hla_bind/) (25). HLA-A2 binding of synthetic peptides was confirmed by T2 stabilization assay as previously described (24).

Generation of dendritic cells. Generation of dendritic cells from peripheral blood monocytes was performed as described previously (23). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and seeded in 6-well plates (Costar, Cambridge, MA) at a concentration of $3x10^6$ cells/well in 3 ml of RPMI-1640 medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with AB+ normal human serum (10%, C-Six Diagnostics, Germantown, WI), L-glutamine (2 mM), HEPES buffer (25 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). After 3 hours at 37°C, 5% CO_2, the non-adherent cells were removed, and the remaining adherent cells were cultured for 7 days in the presence of recombinant human GM-CSF (1000 units/ml) and recombinant human IL-4 (100 ng/ml).

Limiting dilution analysis. The precursor frequency analysis of mammaglobin-reactive proliferating T cells was performed as previously described (26). Briefly, PBMCs were cultured at concentrations of $1x10^4$, $2x10^4$, and $4x10^4$ cells/well (24 wells for each concentration) in round-bottom 96-well plates in the presence of recombinant human IL-2 (50 units/ml, Chiron,

Emeryville, CA) in RPMI-1640 medium supplemented as described above. After 7-10 days, the cells were washed three times and the cultures were resuspended in fresh medium without IL-2. Two thousand irradiated (1750 rads) autologous dentritic cells were added to each well along with recombinant mammaglobin or HA (1μg/well). After 48 hours, the cultures were pulsed with [^{3}H]-thymidine (1 μCi/well). After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. Cultures in the presence of mammaglobin were considered positive when their mean counts per minute (CPM) were three-fold higher than the means CPM of the cultures in the presence of HA (negative control). The results are expressed as the reciprocal of the precursor frequency.

The precursor frequency analysis of mammaglobin-reactive CTLs was performed as previously described (27). Briefly, PBMCs were cultured at concentrations of $1x10^4$, $2x10^4$, and $4x10^4$ cells/well (24 wells for each concentration) in round-bottom 96-well plates in the presence of recombinant IL-2 in RPMI-1640 medium supplemented as described above. After 7-10 days, two thousand ^{51}Cr-labeled (see below), recombinant mammaglobin-pulsed (50μg/ml for 2 hours) or non-pulsed (negative control) autologous dendritic cells were added to each well and incubated for 18 hours. Spontaneous lysis was calculated from target cells incubated culture medium alone and maximum lysis was calculated from target cells incubated in culture medium containing 1% Triton X-100. Specific lysis was calculated according to the following equation: 100x[(experimental ^{51}Cr release–spontaneous ^{51}Cr release)/(maximum ^{51}Cr release–spontaneous ^{51}Cr release)] (27, 28). Cultures in the presence of mammaglobin-pulsed dendritic cells were considered positive when they showed 10% or higher specific lysis as compared to the cultures in the presence of non-pulsed dendritic cells (negative control). The results are expressed as the reciprocal of the precursor frequency.

Generation of CTLs against recombinant mammaglobin and mammaglobin-derived peptides. For CTL induction, $2x10^5$ dendritic cells from a breast cancer patient (HLA-A2,–) were pulsed with recombinant mammaglobin or synthetic mammaglobin-derived peptides (50μg/ml for 2 hours), washed and incubated with $5x10^5$ autologous non-adherent PBMCs in 96-well plates in RPMI-1640 medium supplemented as described above for 7 days. Then, the cultures were stimulated with autologous dendritic cells in the presence of recombinant mammaglobin or peptides and recombinant human IL-2 (10 units/ml) for 5 days. After four stimulations, the CD8+ T cells were purified by negative selection using the mouse IgG monoclonal antibodies against CD4 and CD56 (NK cells) (BD PharMingen, San Diego, CA) and a goat anti-mouse IgG-coated beads (Dynal Inc., Lake Success, NY). The T cells purified in this method were >95% CD8+ as determined by flow cytometric analysis (data not shown). The mammaglobin-specific CTL activity and proliferative response of these CD8+ T cell lines were analyzed five to ten days after the fifth stimulation as described below.

Proliferation assays. In order to measure the mammaglobin-specific proliferative response of the CD8+ T cell lines, triplicate wells containing $5x10^4$ mammaglobin- or peptide-stimulated T cells were cultured in flat bottom 96-well plate in presence or absence of recombinant mammaglobin (10 μg/ml) or cognate peptide (10 μg/ml) along with autologous dendritic cells ($2x10^3$ cells). Irrelevant proteins (HA or BSA, 10 μg/ml) or irrelevant peptide (FK-31, 10 μg/ml) were used as negative controls, respectively. After 48 hours, the cultures were pulsed with [^{3}H]-thymidine (1 μCi/well). After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting.

In order to measure the T cells proliferative response against mammaglobin in healthy adult individuals, PBMC ($2x10^5$/well) from both male and female adult individuals were cultured in quadruplicate cultures in the presence of recombinant mammaglobin (10 μg/ml) or BSA (10

μg/ml) in flat-bottom 96-well plates in RPMI-1640 medium supplemented with as described above. After 4 days, the cultures were pulsed with [^{3}H]-thymidine (1 μCi/well). After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices corresponded to the ratio of the counts per minute of the cultures in the presence of proteins to the mean counts per minute of the cultures in the absence of proteins.

Cytotoxic T lymphocyte activity assay. The standard ^{51}Cr release assay was performed with some modification, as previously described (24). Briefly, autologous dendritic cells were pulsed with recombinant mammaglobin or mammaglobin-derived peptides (50μg/ml for 2 hours). Autologous dentritic cells pulsed with irrelevant protein (HA) or peptide (FK-31) (50μg/ml for 2 hours) were used as negative controls, respectively. Then, the cells were labeled with 250 μCi of ^{51}Cr ($Na^{51}CrO_4$, ICN Pharmeceuticals, Costa Mesa, CA) in 100 μl of RPMI-1640 medium supplemented as described above. After one hour, the labeled cells were plated in triplicate cultures at a concentration of $2x10^3$ cells/well in round bottom 96-well plates. Then, the effector T cells were plated at different concentration as described for each particular experiment. Then, the cultures were incubated at 37°C for 18 hours. In different parallel experiments, selected breast cancer cell lines (Table 1) were labeled and plated as described above and used as target cells in a CTL activity assay. Specific lysis was calculated as described above. For antibody blocking analysis, the target cells were incubated with the W6/32 anti-HLA class I or the KUIA2 anti-HLA-DR monoclonal antibodies (10 μg/ml) for 30 minutes before addition of the effector cells.

Statistical analysis. Statistical analysis of the limiting dilution analysis results was carried out by means of two-tailed unpaired-sample student's T test with the alpha set *a priori* at $P<0.05$.

Results

Lack of mammaglobin-specific T cell proliferative responses in healthy female individuals as compared to healthy male individuals. The expression of mammaglobin has been shown to be restricted to the normal adult mammary epithelium and is overexpressed in 23% of primary breast tumors (10). Therefore, normal individuals would show specific T cell unresponsiveness (tolerance) against mammaglobin. However, the possibility exists that the tissue-specific mammaglobin expression may be regulated by steroid hormones as is the expression of all the uteroglobin family member (11). This hormone-regulated expression of mammaglobin may lead to a differential expression of mammaglobin in the adult mammary epithelium between healthy female and male individuals and hence to differential levels of T cell responsiveness to this same autoantigen. To test this, we compared the T cell proliferative responses to recombinant mammagloblin between healthy adult female and male individuals. As observed in Figure 1, PBMCs derived from healthy adult male individuals showed significantly higher levels of proliferation to mammaglobin as compared to PBMCs from healthy female individuals. Interestingly, no proliferative responses were observed in healthy adult males of females individuals to another autoantigen, HA, which is expressed in comparable levels in individuals from both sexes. These data indicate that adult female individuals may be tolerant to mammaglobin. Therefore, mammaglobin-specific T cells in healthy adult females may be absent from the periphery (clonal deletion) or display antigen-specific unresponsiveness against mammaglobin (anergy).

Higher frequency of mammaglobin-reactive T cells in breast cancer patients than in healthy female individuals. To address the question whether mammaglobin-specific T cells are

deleted or anergized in adult females, we determined the precursor frequency of mammaglobin reactive T cells in healthy adult female individuals and breast cancer patients. If the mammaglobin-specific T cells are anergic, the possibility exists that selective mammaglobin-specific tolerant T cells clones may respond to this protein in case of aberrant expression of the protein as observed in breast tumors. In this case, in a selected group of patients, we would observe a higher frequency of mammaglobin reactive T cells. On the other hand, if the mammaglobin-specific T cell clones have been deleted we would not be able to observe a higher frequency of mammaglobin-reactive T cells in breast cancer patients. As shown in Table 2, the frequencies of mammaglobin-specific proliferating cells in peripheral blood of breast cancer patient ($3.5x10^{-5} \pm 3.2x10^{-5}$) was significantly higher than that observed in healthy female control individuals ($0.15x10^{-5} \pm 0.17x10^{-6}$) ($P=0.03$). Only one of the seven patients included in this study did not respond to recombinant mammaglobin (Table 2, patient No. 7). In addition, as also shown in Table 2, the frequencies of mammaglobin-specific CTLs in peripheral blood of breast cancer patient ($2.4x10^{-5} \pm 1.8x10^{-5}$) was also significantly higher than that observed in healthy female control individuals ($0.39x10^{-5} \pm 0.52x10^{-5}$) ($P=0.03$). Interestingly, one of the seven healthy female control individuals included in this study showed a particularly high CTL precursor frequency (Table 2, control No. 4). These results indicate that, even though healthy female individuals do not respond to mammaglobin as a normal autoantigen, they have mammaglobin-specific T cell clones that expand *in vivo* as result of an aberrant overexpression of this protein in breast tumor cells.

Mammaglobin-specific proliferative response by CD8+ T cell lines generated *in vitro*. Based on the observation that mammaglobin-specific CTLs are normally expanded in breast cancer patients *in vivo*, the possibility existed that high affinity mammaglobin-specific CTL lines could be generated *in vitro*. To test this, we generated a mammaglobin-specific CD8+ T cell line from a breast cancer patient by means of five weekly stimulation *in vitro* as described above. As shown in Figure 2, the resulting T cell line showed a significantly high proliferative response in the presence of recombinant mammaglobin but not in the presence of BSA or HA. This data indicate the mammaglobin-specificity of this reaction.

In parallel experiments, the peptide-specific proliferation of the CD8+ T cell lines generated against the mammaglobin-derived peptides, SKM-67 and SKM-68, was also determined. As shown in Figures 3A and 3B, both of these CD8+ T cell lines demonstrated peptide-specific proliferation upon stimulation with cognate peptide, respectively. In addition, no proliferative response was observed in the presence of the irrelevant FK31 HLA-A3-binding peptide. Since both mammaglobin-derived peptides (SKM-67 and SKM-68) shared the common anchor residues (L in position 2 and A in position 9), proliferation was observed in reciprocal combination, although the responses were weaker as compared to the corresponding cognate peptide. Overall, these data indicate that mammaglobin-specific CD8+ T cell lines can be developed *in vitro*.

Mammaglobin-specific CTL activity by CD8+ T cell lines generated *in vitro*. The results shown above indicate the specific reactivity against mammaglobin of three different CD8+ T cell lines generated *in vitro*. To analyze the mammaglobin-specific CTL activity of the CD8+ T cell line generated against recombinant mammaglobin, we determined the ability of these CD8+ T cells to lyse autologous dendritic cells pulsed with recombinant mammaglobin. As shown in Figure 4, this particular CTL line demonstrated antigen-specific killing of recombinant mammaglobin-pulsed autologous dendritic cells. No lytic activity was observed with non-pulsed autologous dendritic cells as well as autologous dendritic cells pulsed with an irrelevant protein (HA). The lytic activity observed with the mammaglobin-pulsed dendritic cells was significantly inhibited by pre-treatment with W6/32, an anti-HLA-class I monoclonal antibody (60%

inhibition) but not with KUIA-2, an anti-HLA-DR monoclonal antibody (12% inhibition). These data demonstrate the HLA class I-restricted recognition of mammaglobin-derived epitopes by this CTL line.

As shown in the Figure 5A and 5B, CD8+ T cell lines obtained in vitro against mammaglobin-derived peptides (SKM-67 and SKM-68) also show a significant CTL activity against T2 cells (HLA-A2-positive, Tap-deficient cell line) pulsed with the corresponding cognate peptide. Peptide-specific CTL activity was also observed with peptide-pulsed autologous dendritic cells, although the percent of lysis was lower as compared to the one observed with peptide-pulsed T2 cells (Figure 5) or to dendritic cells pulsed with the whole recombinant protein (Figure 4) (data not shown). This result indicates that both SKM-67 and SKM-68 are recognized in the context of HLA-A2 the CTL lines generated *in vitro.*

Mammaglobin-specific MHC-restricted CTL activity on breast cancer cells by CD8+ T cells generated *in vitro*. After five weekly stimulations with recombinant mammaglobin, a CTL line from a HLA-A2-positive breast cancer patient was tested for their ability to lyse several breast cancer cell lines naturally expressing the mammaglobin protein in the context of HLA-A2 (Table 1). As shown in Figure 6, this CTL line selectively killed mammaglobin-positive breast cancer cell lines in the context of HLA-A2 (HBL-100 and AU565). No killing was observed with mammaglobin-positive, HLA-A2-negative breast cancer cell lines (MDA-MB-415 and MDA-MB-361) or mammaglobin-negative, HLA-A2-positive breast cancer cell lines (MCF-7 and MDA-MB-231). No NK cell activity was observed against K562, an NK cell-sensitive cell line. These results clearly demonstrate the MHC restriction and antigen specificity of the CTL activity mediated by mammaglobin-specific CTLs generated *in vitro*.

Discussion

Breast cancer accounts for 30% of all cancer diagnosed in US (29). Among the genetic alterations identified in human breast cancer, one of the most important is the increased activity of the ERBB2 gene family (30). Amplification of ERBB2 occurs in 30-40% of early stage breast cancer, and a significant correlation between ERBB2 over-expression and reduced survival of breast cancer patients has been documented (30). Besides the ERBB2, germline mutations in BRCA1 and BRCA2 genes and other rare variants accounts for 15-20% of breast cancer that clusters in families and less than 5% of breast cancer overall (31). In addition to these tumor-associated markers, the aberrant overexpression of the mammaglobin gene has been recently described in primary and metastatic breast tumor and in the breast cancer cells present in the peripheral circulation (10, 17-19). It has been shown that approximately 80% of all primary and metastatic breast tumors are strongly immunopositive for mammaglobin protein and staining was independent of tumor grade (20). In this manuscript, we investigated whether mammaglobin-specific T cells are present in healthy individuals as well as in breast cancer patients. Further, we also investigated the feasibility to develop mammaglobin-specific CTL lines *in vitro* with the capacity to lyse breast cancer cells in an MHC-restricted manner.

It is well-established that CD8+ T cells recognize peptide antigens of 8-12 amino acid long that are presented in the context of MHC class I molecules on antigen presenting cells (32-35). In this regard, CTLs are a critical component of host immunity to tumors since they have been shown to recognize tumor-associated antigens in an MHC-restricted manner on several tumor cells (36). Therefore, definition of CTL epitopes is an important area for the development of CTL-based cancer immunotherapies. Dendritic cells have been shown to be prime naïve resting CTL when they are pulsed either with antigenic peptides or proteins (37-39). More

recently, effective tumor immunity in mice was induced using dendritic cells pulsed with unfractionated tumor-derived antigens in the form of peptides, cell sonicates, or messenger RNA (mRNA) (38,40,41). Several investigators have generated peptide-specific CTLs against a variety of oncogenic protein like Her-2/neu or MAGE restricted by HLA-A2 or HLA-A3 molecules (5,6).

Our study indicates that breast cancer patients have higher frequency of mammaglobin-reactive proliferating T cells as well as CTLs as compared to healthy individuals (Table 2). These observations are the first demonstration of mammaglobin-specific T cells circulating in the peripheral blood of breast cancer patients. The low frequency of circulating mammaglobin-reactive T cells in healthy female individuals was further supported by the lack of proliferative response of total PBMCs to recombinant mammaglobin as compared to healthy male individuals. As mentioned above, it is possible that mammaglobin-reactive T cells from normal female individuals are either anergic or deleted since mammaglobin is a self antigen. High proliferative response in breast cancer patients to mammaglobin could be attributed to the reemergence of mammaglobin-reactive T cells by a break of self tolerance. It would be of interest to know whether there is any correlation between various stages of breast cancer and a detectable increase in proliferative and/or CTL responses to mammaglobin in breast cancer patient.

To determine whether peripheral blood T cells from breast cancer patients could also generate CTL response *in vitro*, we generated a CTL line from a breast cancer patient using autologous dendritic cells pulsed with recombinant mammaglobin or mammaglobin-derived, HLA-A2-restricted synthetic peptides. Our result indicates that mammaglobin-specific CTLs have the ability to kill autologous dendritic cells expressing naturally processed mammaglobin-derived epitopes (Figure 4). We also identified two HLA-A2-restricted epitopes (SKM-67, SKM-68) using autologous dendritic cells pulsed with synthetic peptides. CTLs generated against these two peptides showed enhanced lysis of T2 cells pulsed with the corresponding cognate peptide (Figures 5A and 5B). This peptide-specific CTL lines also lysed autologous dendritic cells pulsed with the corresponding cognate peptide (data not shown). Further, we tested the cytotoxic potential of the mammaglobin-reactive CTL line against breast cancer cells. We show herein that mammaglobin-specific CTLs generated from a breast cancer patient also have the capacity to lyse mammaglobin-positive breast cancer cells in an MHC-restricted manner. More importantly, these data indicate that peptides derived from mammaglobin are naturally processed and presented in breast cancer cells in the context of MHC class I molecules. Further, CTLs generated against whole mammaglobin protein could lyse breast cancer cells expressing mammaglobin with the appropriate MHC class I restriction element.

In summary, we have described the generation of T cell responses specific to mammaglobin and mammaglobin-derived HLA-A2-restricted peptide epitopes in breast cancer patients. Limiting dilution analyses revealed that breast cancer patients have higher frequency of mammaglobin-reactive proliferating cells as well as CTLs in peripheral blood as compared to normal female individuals. These results shows that dendritic cells pulsed with mammaglobin or mammaglobin-derived peptide could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

References

1. Porter-Jordan K and Lippman ME. Overview of the biologic markers of breast cancer. Hematol Oncol Clin N Am 1994;8:73-100.
2. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, and Boon T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 1995;182:689-98.
3. Berard F, Blanco P, Davoust J, Neidhart-Berard EM, Nouri-Shiraji M, Taquet N, Rimoldi D, Cerottini JC, Banchereau J, and Palucka AK. Cross priming of naïve CD8+ T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. J Exp Med 2000;192:1535-44.
4. Peoplles GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, and Eberlein TJ. Breast and ovarian cancer specific cytotoxic T lymphocytes recognize the same HER-2/neu-derived peptide. Proc Natl Acad Sci USA 1995;92:432-36.
5. Brossart P, Stuhler G, Flad T, Stevanovic S, Rammensee H-G, Kanz L, and Brugger W. Her-2/neu derived peptides are tumor associated antigens expressed by human renal cell and colon carcinomalines and are recognized by in vitro induced specific cytotoxic T lymphocytes. Cancer Res 1998;58:732-36.
6. Kawashima I, Tsai V, Southwood S, Takesako K, Sette A, and Celis E. Identification of HLA-A3 restricted cytotoxic T lymphocyte epitopes from carcinoembryonic antigen and Her-2/neu by primary in vitro immunization with peptide pulsed dendritic cells. Cancer Res 1999;59:431-35.
7. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ulrich A, and Press MF. Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. Science 1989;244:707-12.
8. Welcsh PL, and King MC. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Hum Mol Genet 2001;10:705-13.
9. Watson MA, and Fleming TP. Isolation of differentially expressed sequence tags from human breast cancer. Cancer Res 1994;54:4598-4602.
10. Watson MA, Fleming TP. Mammaglobin, a mammary specific member of the uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res 1996;56:860-65.
11. Watson MA, Darrow C, Zimonjic DB, Popescu NC, and Fleming TP. Structure and Transcriptional regulation of the human mammaglobin gene, a breast cancer associated member of the uteroglobin gene family localized to chromosome 11q13. Oncogene.1998;16:817-24.
12. Forsgren B, Bjork P, Carlstrom K, Gustafsson JA, Pousette A, and Hogberg B. Purification and distribution of major protein in rat prostate that binds estramustine, a nitrogen mustard derivative of estradiol-17B. Proc Natl Acad Sci USA 1979;76:3149-53.
13. Oscar LA, Petrusz P, and French FS. Prostatein. A major secretory protein of rat ventral prostate. J Biol Chem 1979;254:6196-6202.
14. Miele L, Miele EC, Mantile G, Peri A, and Mukherjee AB. Uteroglobin and Uteroglobin like proteins: The uteroglobin family of proteins. J Endocrinol Invest 1994;17:679-92.
15. Paik S, Burkhard E, and Lippman ME. Clinical significance of erb/b2 protein overexpression. Cancer Treat Res 1992;61:181-91.
16. Keyomarsi K, and Pardee AB. Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc Natl Acad Sci USA 1993;1112-16.
17. Zach O, Kasparu H, Krieger O, Hehenwarter W, Girschikofsky M, and Lutz D. Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients via nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. J of Clin Onc 1999;17:2015-19.

18. Grunewald K, Haun M, Urbanek M, Fiegel M, Muller-Holzner E, Gunsilius E, Dunser M, Marth C, and Castle G. Mammaglobin gene expression: a superior marker of breast cancer cells in peripheral blood in comparison to epidermal growth factor receptor and cytokeratin-19. Lab Invest 2000;80:1071-77.
19. Suchy B, Austrup F, Driesel G, Eder C, Kusial I, Uciechowski P, Grill H-J, and Giesing M. Detection of mammaglobin expressing cells in blood of breast cancer patients. Cancer Letters 2000;158:171-78.
20. Min CJ, Tafra L, and Verbanac KM. Identification of superior markers for polymerase chain reaction detection of breast cancer metastases in sentinel lymph nodes. Cancer Research 1998;58:4581-84.
21. Watson MA, Dintzis S, Darrow CM, Voss LE, DiPersio J, Jensen R, and Fleming TP. Mammaglobin expression in primary, metastatic and occult breast cancer. Cancer Research 1999;59:3028-31.
22. Leygue E, Snell L, Dotzlaw H, Holes K, Troup S, Hiller-Hitchcock T, Murphy LC, and Watson PH. Mammaglobin, a potential marker of breast cancer nodal metastasis. J Pathol 1999:189:28-33.
23. Zhou JL, and Tedder TF. CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. Proc Natl Acad Sci USA 1996;93:2588-92.
24. Poindexter NJ, Naziruddin B, McCourt DW, and Mohanakumar T. Isolation of a kidney specific peptide recognized by alloreactive HLA-A3 restricted human CTL. J Immunol 1995;154:3880-87.
25. Parker KC, Bednarek MA, and Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side chains. J Immunol 1994;152:163-175.
26. Liu Z, Colovai AI, Tugulea S, Reed EF, Fisher PE, Mancini D, Rose EA, Cortesini R, Michler RE, and Suciu-Foca N. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J Clin Invest 1995;98:1150-57.
27. Goulder JRP, Tang Y, Brander C, Betts MR, Altfels M, Annamalai K, Trocha A, He S, Rosenberg ES, Ogg G, O'Callaghan CA, Kalamas SA, McKinney Jr, RE, Mayer K, Koup RA, Pelton SI, Burchett SK, McIntosh K, and Walker B. Functionally Inert HIV specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children. J Exp Med 2000;192:1819-31.
28. de St. Growth F. The evaluation of limiting dilution assays. J Immunol Method 1982;49:11-23.
29. Greenlee RT, Murray T, Bolden S, and Wingo PA. Cancer Statistics, 2000. CA Cancer J Clin 2000;50:7-33.
30. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ulrich A, and McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177-182.
31. Peto J, Collins N, Barfoot R, Seal S, Warren W, Rahman N, Easton DF, Evans C, Deacon J, and Stratton MR. Prevalence of BRCA1 and BRCA2 gene mutations in patients with early–onset breast cancer. J Natl Cancer Inst 1999;91:943-49.
32. Rammensee HG, Falk K, and Rotzschke O. Peptides naturally presented by MHC class I molecules. Ann Rev Immunol 1993;11:231-44.
33. Bevan MJ. Antigen presentation to cytotoxic T lymphocytes in vivo. J Exp Med 1995;182:639-41.
34. York IA, and Rock KL. Antigen processing and presentation by the class I major histocompatibility complex. Ann Rev Immunol 1996;14:369-96.
35. Zinkernagel RM, and Doherty PC. The discovery of MHC restriction. Immunol Today 1997;18: 14-17.

36. Berke G. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. Ann Rev Immunol 1994;12:735-73.
37. Cella M, Sallusto F, and Lanzavecchia L. Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 1997;9:10-16.
38. Zitovogel L, Mayordoma JI, Storkus WJ, Lotze MT, and Falo LD. Therapy of murine tumors with tumor peptide pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell associated cytokines. J Exp Med 1996;183:87-97.
39. Pagalia P, Chiodoni C, Rodolfo M, and Colombo MP. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med 1996;183:317-22.
40. Ashley DM, Faiola B, Nair S, Hale LP, Bigner DD, and Gilboa E. Bone marrow generated dendritic cells pulsed with tumor extract or tumor RNA induce antitumor immunity against central nervous system tumors. J Exp Med 1997;186:1177-82.
41. Nair SK, Heiser A, Boczkowski D, Majumder A, Naoe M, Lebkowski JS, Vieweg J, and Gilboa G. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. Nature Medicine 2000;6:1011-17.

Notes

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Table 1
Breast cancer cell lines used in this study

Breast Cancer Cell Line	Tumor Classification	HLA-A Phenotype	Mammaglobin Expression
MCF-7	Adenocarcinoma	HLA-A2,–	–
MDA-MB-231	Adenocarcinoma	HLA-A2, –	–
HBL-100	Adenocarcinoma	HLA-A1,2	+
AU-565	Adenocarcinoma	HLA-A2,3	+
MDA-MB-361	Adenocarcinoma	HLA-A1,24	+
MDA-MB-415	Adenocarcinoma	HLA-A30,33	+

Table 2
Frequency of mammaglobin-reactive T cells in peripheral blood of breast cancer patients and healthy female individuals

Study Subjects	Frequency of Proliferating T cells[1,2]	Frequency of CTLs[1,3]
Patients		
01	9.00×10^{-5}	1.10×10^{-5}
02	3.31×10^{-5}	5.98×10^{-5}
03	2.74×10^{-5}	1.84×10^{-5}
04	1.07×10^{-5}	3.36×10^{-5}
05	1.88×10^{-5}	4.80×10^{-6}
06	6.73×10^{-5}	2.06×10^{-5}
07	0	2.02×10^{-5}
Mean ± SD:	$3.5 \times 10^{-5} \pm 3.2 \times 10^{-5}$	$2.4 \times 10^{-5} \pm 1.8 \times 10^{-5}$
Controls		
01	0	8.00×10^{-7}
02	4.00×10^{-7}	0
03	1.70×10^{-6}	5.00×10^{-6}
04	1.30×10^{-6}	1.50×10^{-5}
05	5.10×10^{-6}	3.60×10^{-6}
06	1.40×10^{-6}	1.10×10^{-6}
07	8.00×10^{-7}	1.90×10^{-6}
Mean ± SD:	$1.50 \times 10^{-6} \pm 1.70 \times 10^{-6}$	$3.90 \times 10^{-6} \pm 5.20 \times 10^{-6}$

[1]Results are expressed as the reciprocal of the precursor frequency.
[2]Frequency of proliferating T cells: Patients vs. Controls, $P = 0.03$ (two-tailed student's T test).
[3]Frequency of CTLs: Patients vs. Controls, $P = 0.03$ (two-tailed student's T test).

Figure Legends

Figure 1. Lack of mammaglobin-specific T cell proliferative responses in healthy female individuals as compared to healthy male individuals. PBMC from both healthy male (n=6) and female (n=6) adult individuals were cultured in the presence of recombinant mammaglobin or BSA in flat-bottom 96-well plates. After 4 days, the cultures were pulsed with [^{3}H]-thymidine. After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results expressed as stimulation indices ± standard deviations corresponded to the ratio of the counts per minute of the cultures in the presence of proteins to the mean counts per minute of the cultures in the absence of proteins (negative control). Results shown are representative of 3 different experiments.

Figure 2. Mammaglobin-specific proliferative response by CD8+ T cells generated *in vitro* against recombinant mammaglobin. Non-adherent PBMCs from a breast cancer patient were stimulated weekly with autologous dendritic cells pulsed with recombinant mammaglobin. After four stimulations, the CD8+ T cells were purified by negative selection and the proliferative response to mammaglobin was analyzed five days after the fifth stimulation. The CD8+ T cells were cultured in the presence or absence of recombinant mammaglobin, BSA, or HA in flat-bottom 96-well plates in the presence of autologous dendritic cells. After 48 hours, the cultures were pulsed with [^{3}H]-thymidine. After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. Results are expressed as the mean counts per minute ± standard deviations of triplicate cultures.

Figure 3. Peptide-specific proliferative response by CD8+ T cells generated *in vitro* against mammaglobin-derived peptides. Non-adherent PBMCs from a breast cancer patient were stimulated weekly with autologous dendritic cells pulsed with mammaglobin-derived peptides (SKM-67 and SKM-68). After four stimulations, the CD8+ T cells were purified by negative selection and the proliferative responses to mammaglobin-derived peptides were analyzed ten days after the fifth stimulation. The CD8+ T cells were cultured in the presence or absence of the SKM-67 and SKM-68 mammmaglobin-derived peptides and the FK-31 irrelevant control peptide in flat-bottom 96-well plates in the presence of autologous dendritic cells. After 48 hours, the cultures were pulsed with [^{3}H]-thymidine. After 18 hours, [^{3}H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. Panels A and B show the proliferative responses of the anti-SKM-67 and anti-SKM-68 CD8+ T cell lines, respectively. Results are expressed as the mean counts per minute ± standard deviations of triplicate cultures.

Figure 4. Mammaglobin-specific CTL activity by CD8+ T cells generated *in vitro* against recombinant mammaglobin. Non-adherent PBMCs from a breast cancer patient were stimulated weekly with autologous dendritic cells pulsed with recombinant mammaglobin. After four stimulations, the CD8+ T cells were purified by negative selection and the mammaglobin-specific CTL activity was analyzed five days after the fifth stimulation. Briefly, autologous dendritic cells were pulsed with recombinant mammaglobin or HA. Then, the cells were labeled with ^{51}Cr and plated in round bottom 96-well plates. Then, the effector T cells were plated at a concentration of $50x10^3$/well (effector:target ratio: 25:1). After 18 hours of incubation, the specific lysis was calculated as described in materials and methods. Results are expressed as the mean percentages of specific lysis ± standard deviations of triplicate cultures. For antibody blocking analysis, the target cells were incubated with the W6/32 anti-HLA class I or the KUIA2 anti-HLA-DR monoclonal antibodies for 30 minutes before addition of the effector T cells.

Figure 5. Peptide-specific CTL activity by CD8+ T cells generated *in vitro* against mammaglobin-derived peptides. Non-adherent PBMCs from a breast cancer patient were stimulated weekly with autologous dendritic cells pulsed with mammaglobin-derived peptides (SKM-67 and SKM-68). After four stimulations, the CD8+ T cells were purified by negative

selection and the peptide-specific CTL activity was analyzed ten days after the fifth stimulation. Briefly, autologous dendritic cells were pulsed with the correlponding cognate mammaglobin-derived peptides. Non-pulsed dendritic cells were used as negative controls. Then, the cells were labeled with ^{51}Cr and plated in round bottom 96-well plates. Then, the effector T cells were plated at concentration of $100x10^3$, $50x10^3$, $25x10^3$, and $12.5x10^3$ (effector:target ratios: 50:1, 25:1, 12.5:1 and 6.25:1, respectively). After 18 hours of incubation, the specific lysis was calculated as described in materials and methods. Panels A and B show the CTL activities of the anti-SKM-67 and anti-SKM-68 CD8+ T cell lines, respectively. Results are expressed as the mean percentages of specific lysis ± standard deviations of triplicate cultures.

Figure 6. Mammaglobin-specific MHC-restricted CTL activity on breast cancer cells by CD8+ T cells generated *in vitro* against recombinant mammaglobin. Non-adherent PBMCs from a breast cancer patient were stimulated weekly with autologous dendritic cells pulsed with recombinant mammaglobin. After four stimulations, the CD8+ T cells were purified by negative selection and the mammaglobin-specific CTL activity was analyzed five days after the fifth stimulation. Briefly, breast cancer cells were labeled with ^{51}Cr and plated in round bottom 96-well plates. Then, the effector T cells were plated at a concentration of $10x10^3$/well (effector:target ratio: 5:1). After 18 hours of incubation, the specific lysis was calculated as described in materials and methods. Results are expressed as the mean percentages of specific lysis ± standard deviations of triplicate cultures.

Figure 1

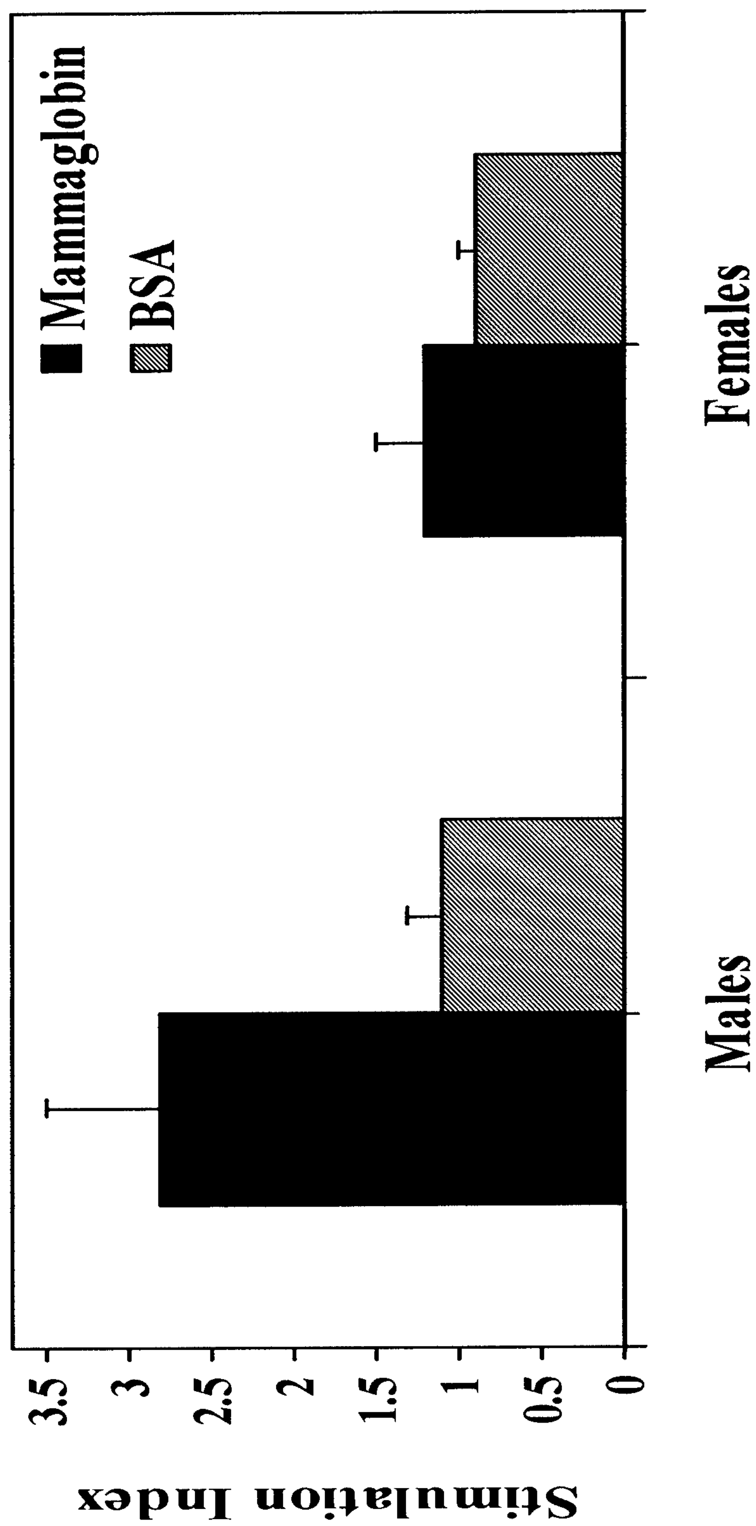


Figure 2

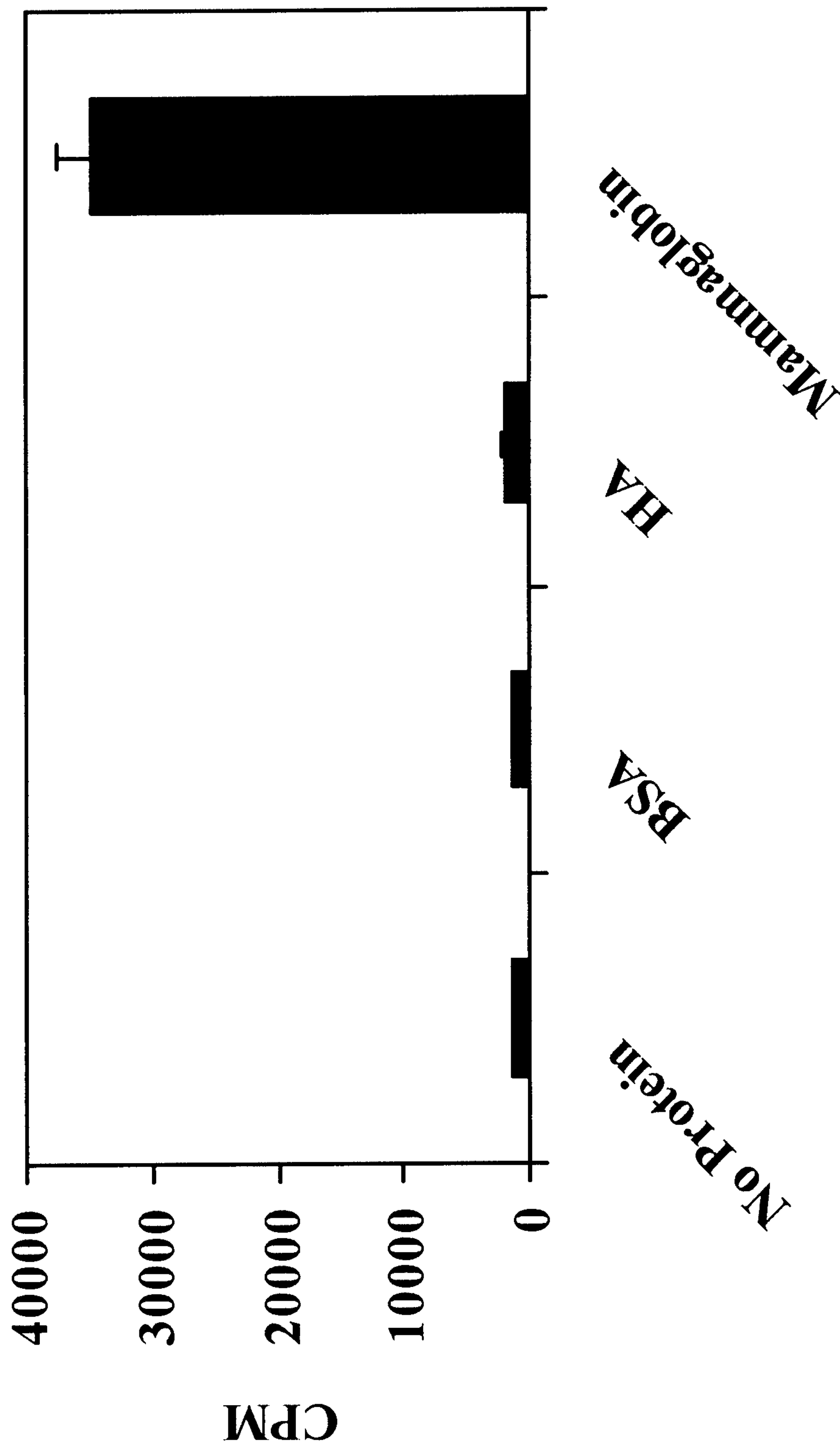
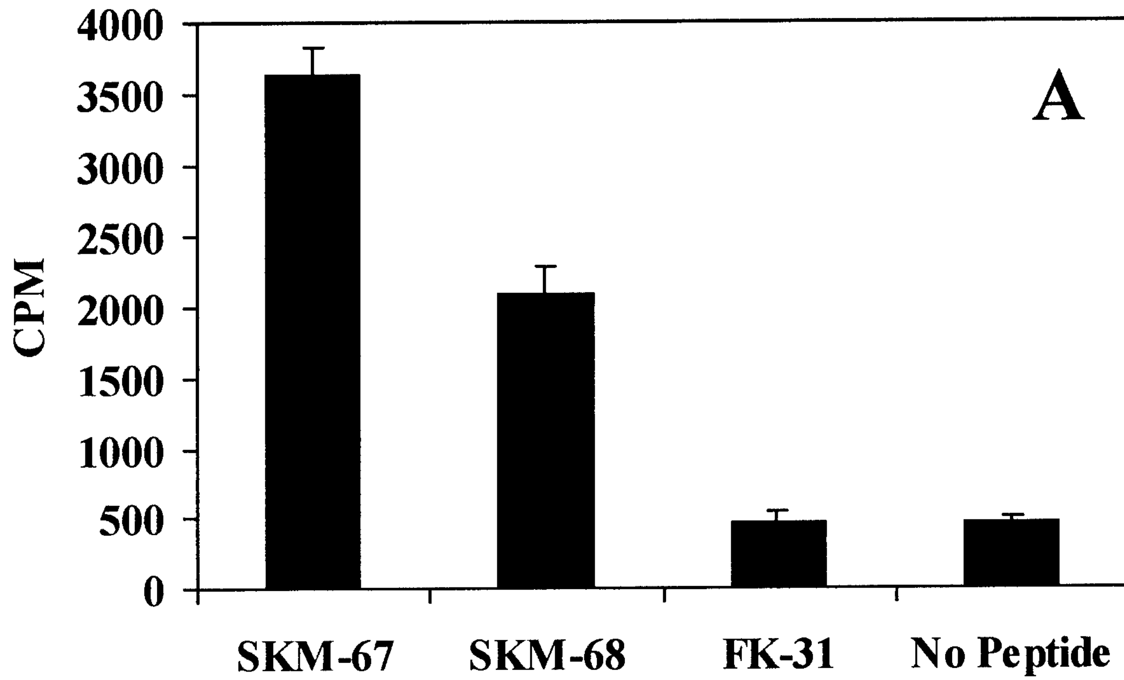


Figure 3



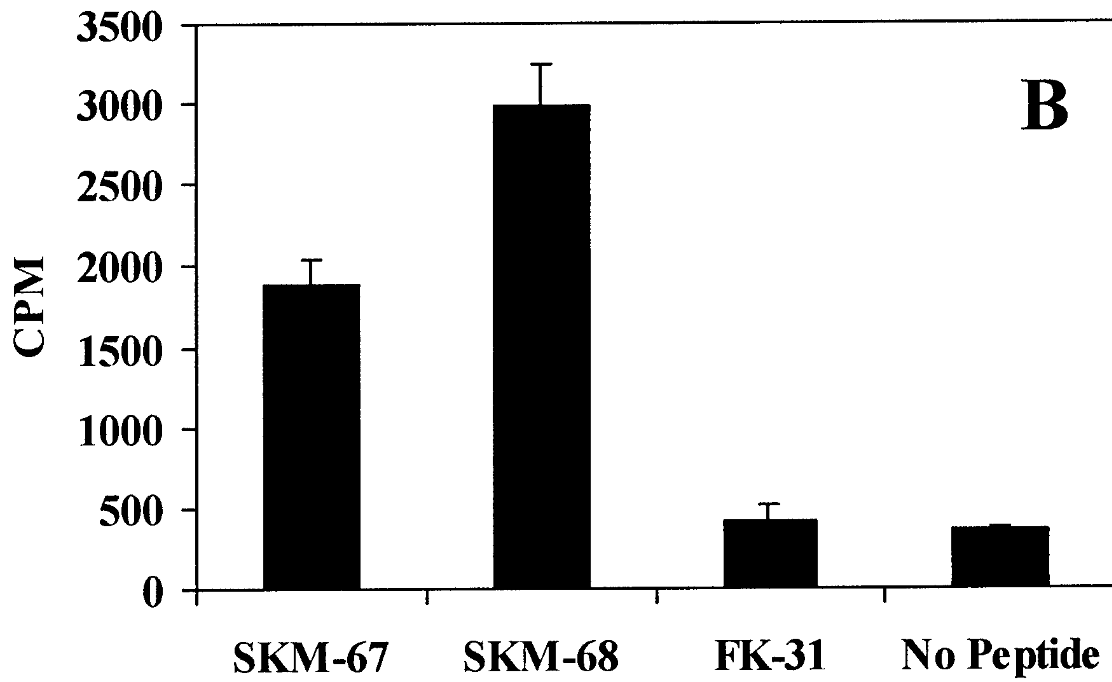


Figure 4

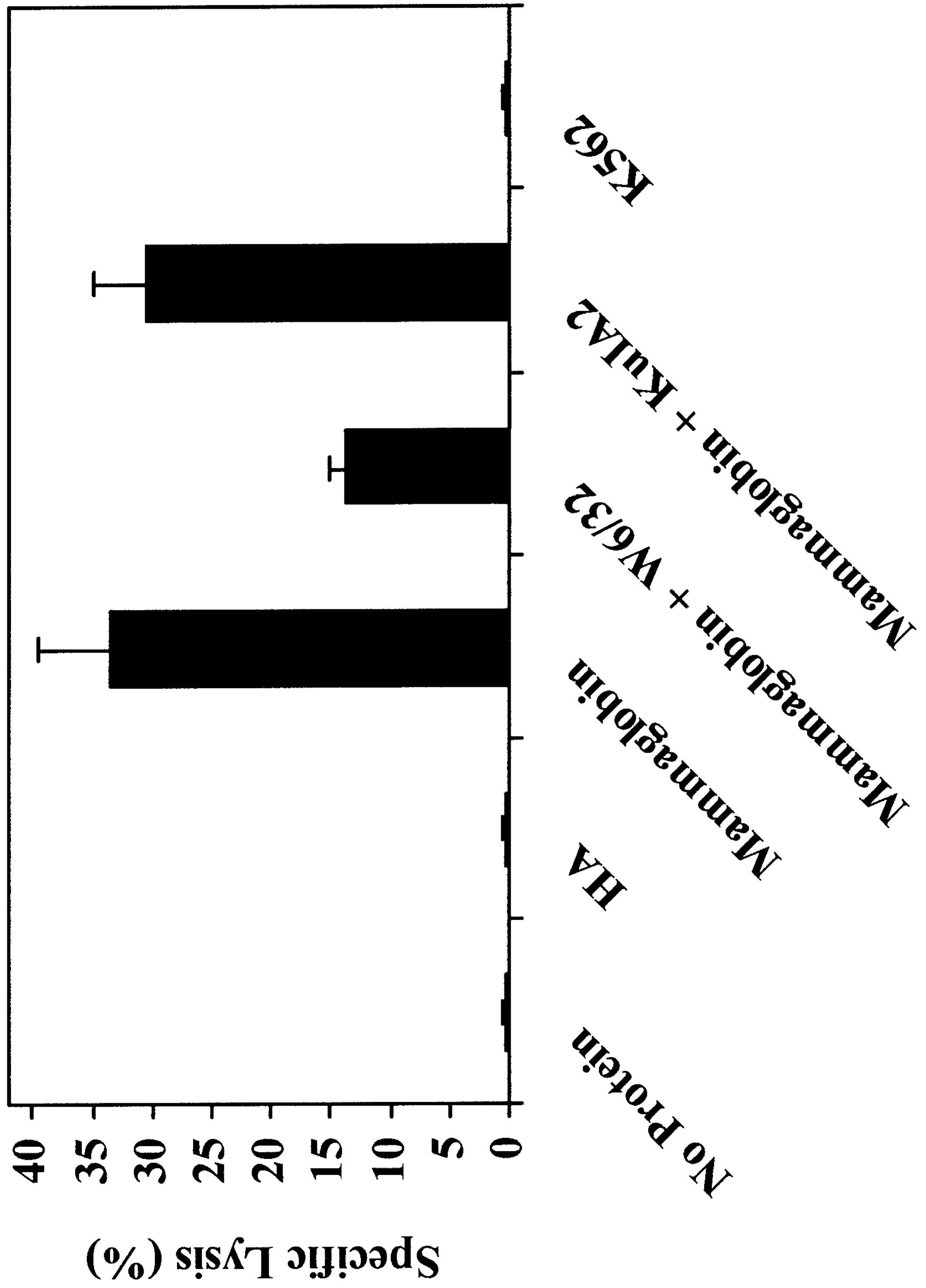
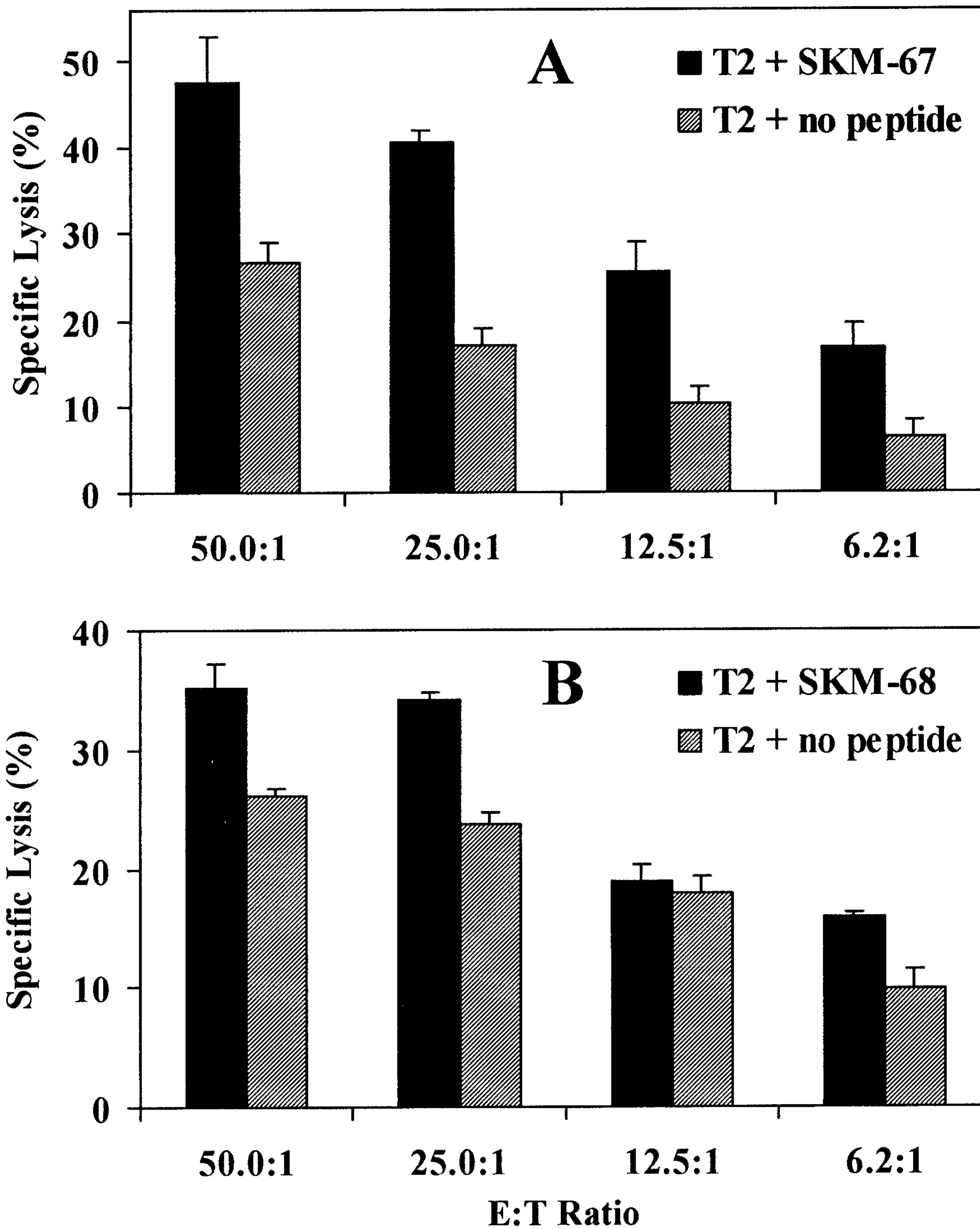


Figure 5



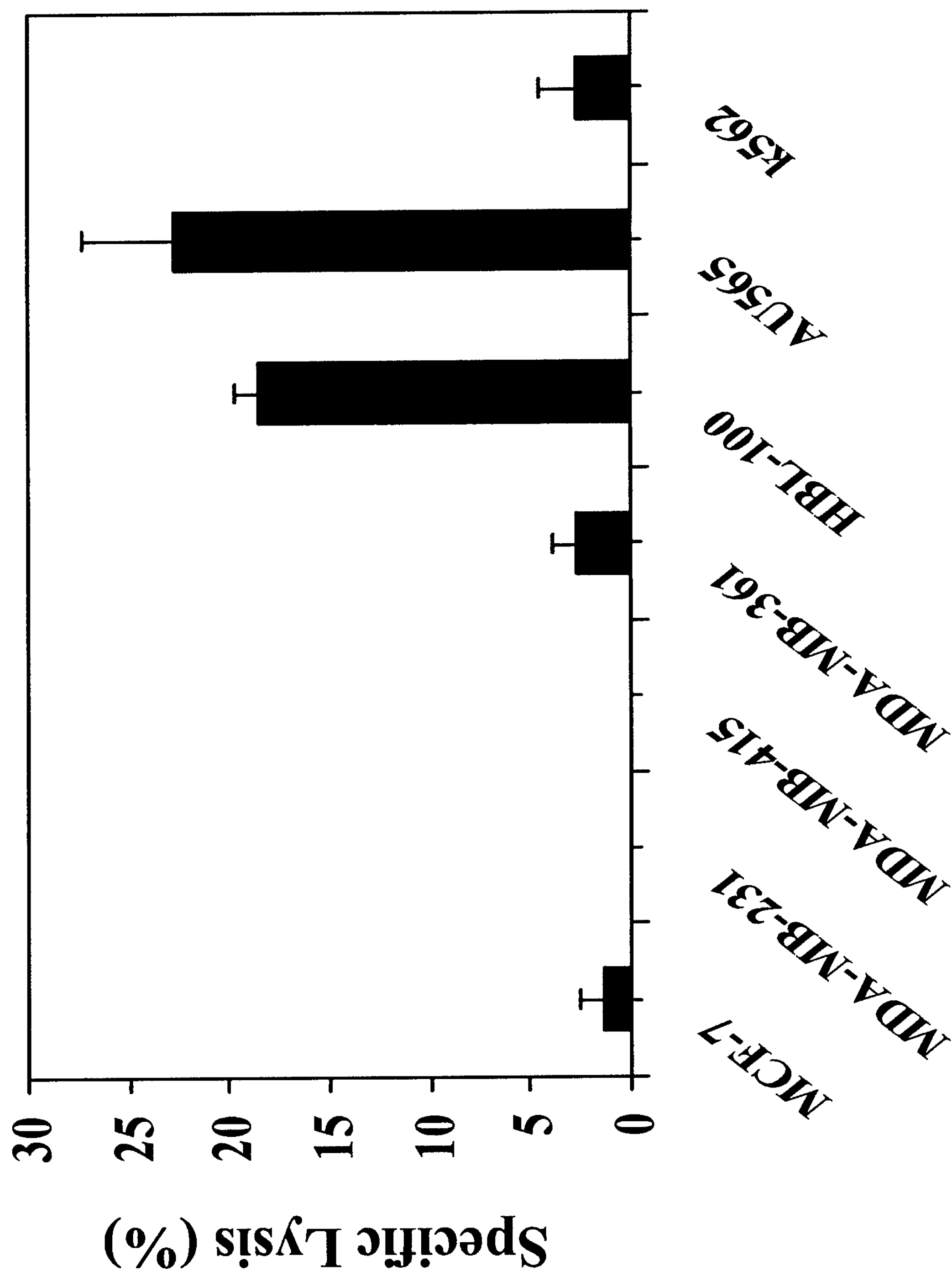


Figure 6
Specific Lysis (%)
0
5
10
15
20
25
30
MCF-7
MDA-MB-231
MDA-MB-415
MDA-MB-361
HBL-100
AU565
k562

Table 1
Breast Cancer Cell lines

Breast Cancer Cell Line	Tumor Classification	HLA-A Phenotype	Mammaglobin Expression
DU-4475	Adenocarcinoma	HLA-A3,31	Positive
AU-565	Adenocarcinoma	HLA-A2,3	Positive
HBL-100	Adenocarcinoma	HLA-A1,2	Positive
T-47D	Ductal Carcinoma	HLA-A3,26	Negative
MCF-7	Adenocarcinoma	HLA-A2,–	Negative

Figure 1
CD8+ CTLs Generated In Vitro Against Mammaglobin-Derived Peptides Recognize a Single Immunodominant Epitope

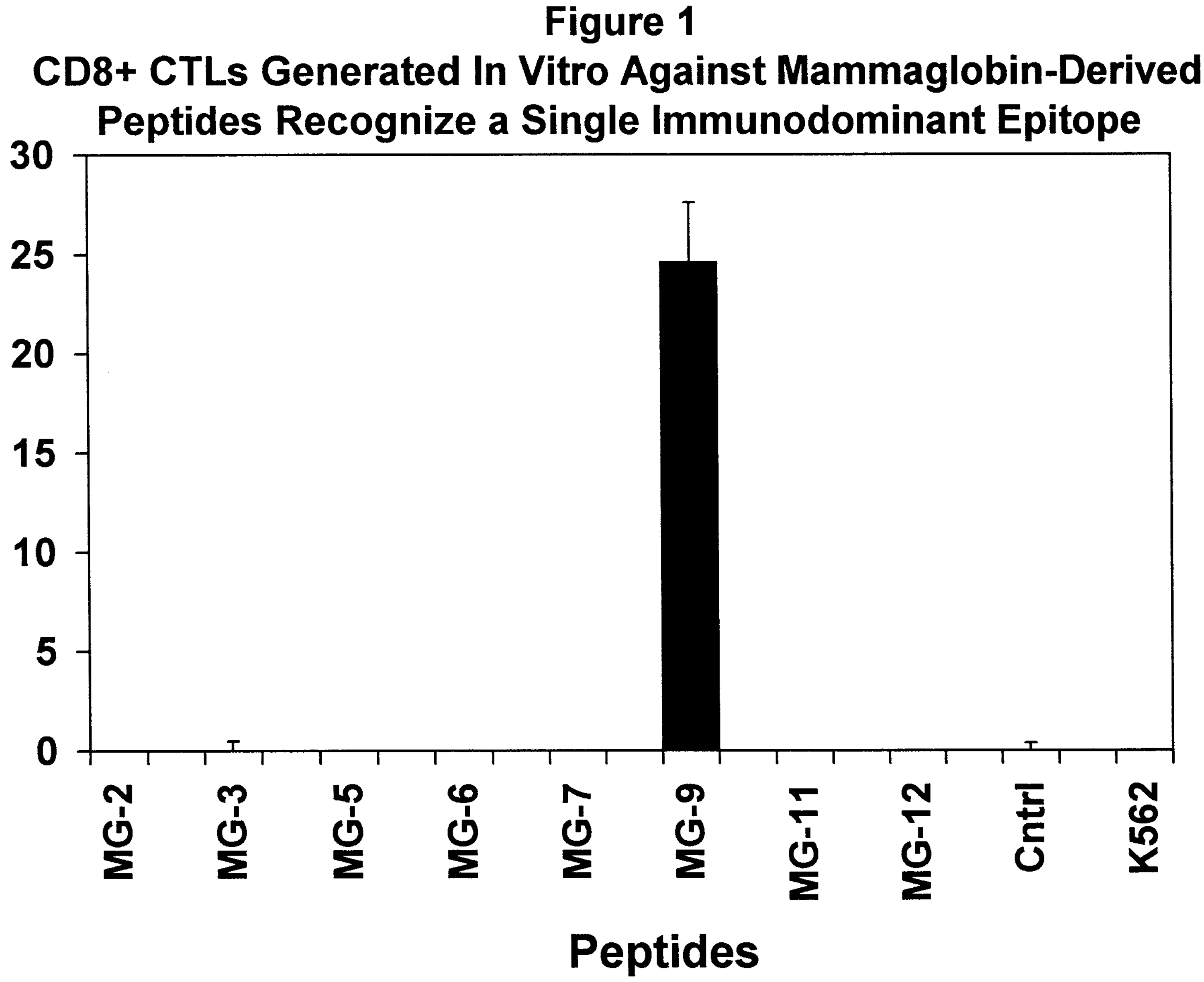


Figure 2
Cytotoxic Activity on Breast Cancer Cells of the Anti-MG-9 CD8+ CTL Line

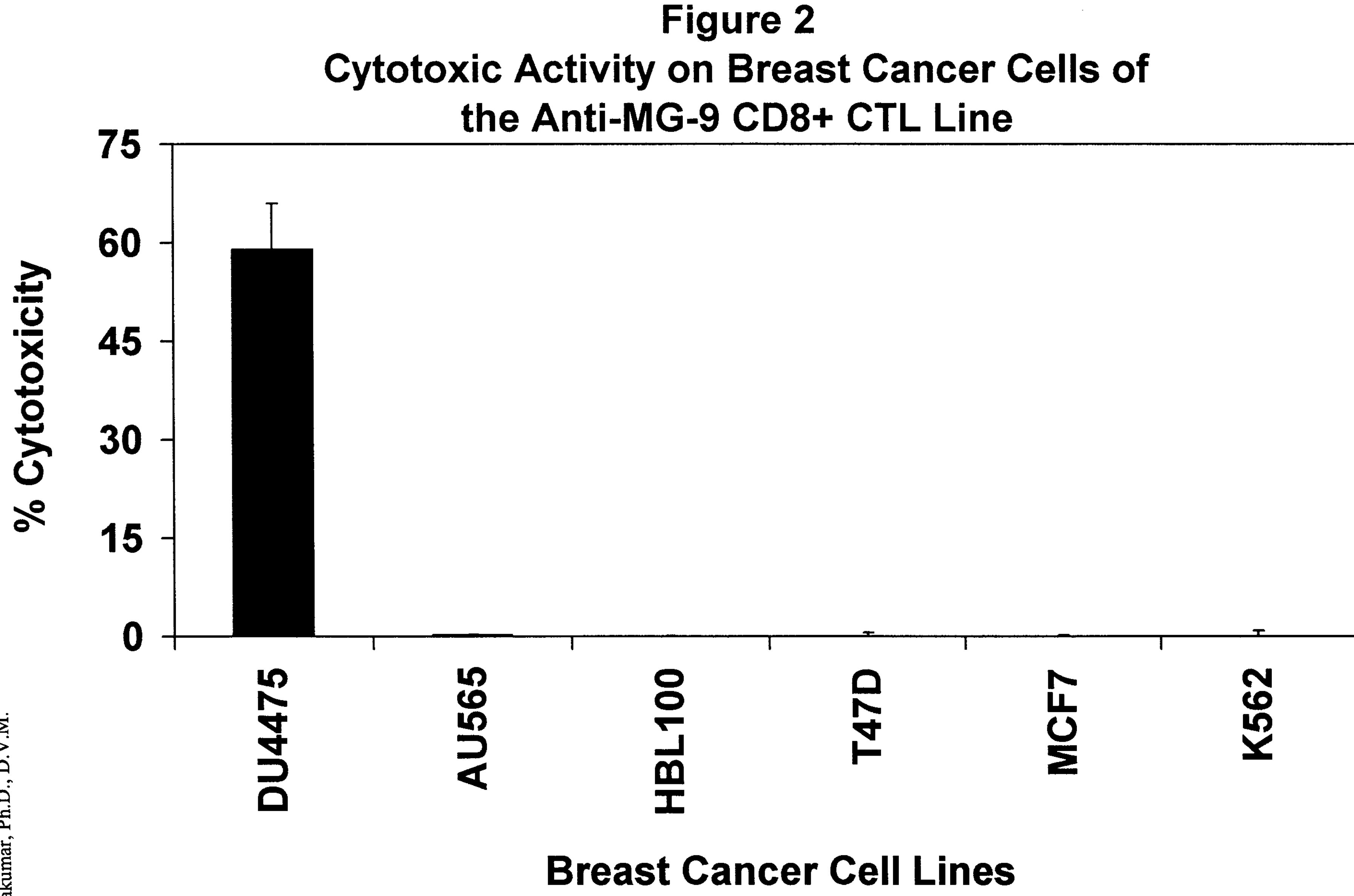


Figure 3
In Vivo Response to HLA-A2-Binding Mammaglobin-Derived Peptides

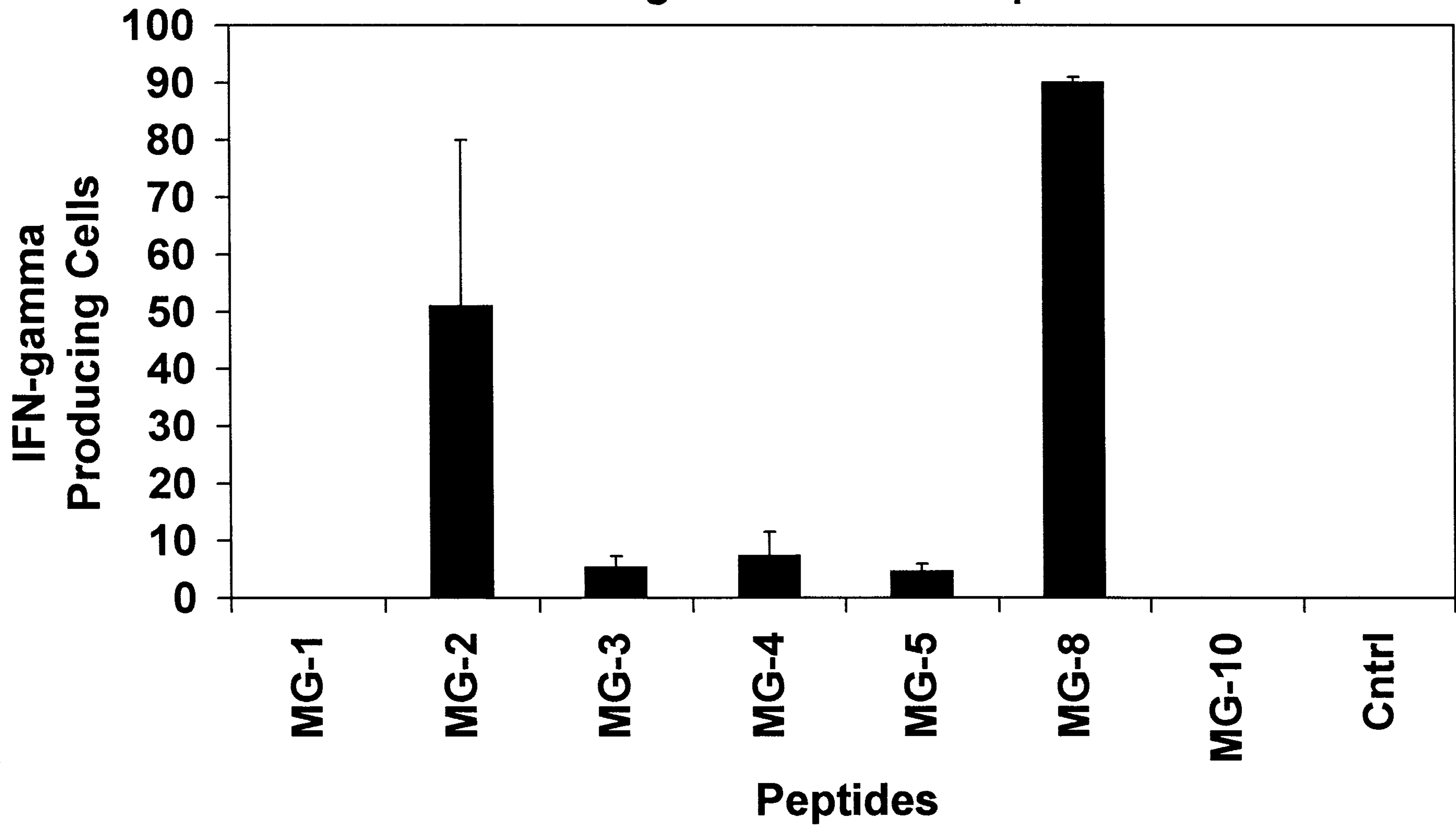


Figure 4
In Vivo Response to HLA-A3-Binding Mammaglobin-Derived Peptides