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ABSTRACT

This project is focused on novel tumor vaccines directed at MUC1 and other tumor antigens. Our specific aims are: 1)To assess the effectiveness of vaccines against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice; 2)To translate an effective vaccine strategy into a phase I clinical trial in patients with undetectable disease following standard therapy. The model of spontaneous mammary cancer is the MUC1-expressing polyoma middle T antigen mice (MMT). We have tested five vaccines in the preclinical mouse model and all elicited a strong immune response. The vaccine using MUC1 class I binding peptides prevented MUC1-expressing tumor growth. We have designed the Phase I clinical trial using a peptide vaccine comprised of MUC1 and HER-2/neu MHC class I peptides and HER-2/neu MHC class II peptide with unmethylated CpG oligodeoxynucleotides and GM-CSF as adjuvants. The clinical trial has been unanimously approved by the Mayo Institutional Review Board (IRB 582-05) following receipt of FDA approval (BB-IND 12155). The peptides have been synthesized and vialed. It is a phase I trial testing MUC1 and HER-2/neu class I and class II peptides with CpG ODN and GM-CSF adjuvants in breast cancer patients free of disease. The clinical trial documents were formally submitted in May 2005 for USAMRMC approval.

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INTRODUCTION

This project is focused on the development of novel tumor vaccines directed at MUC1, a transmembrane mucin that is aberrantly expressed in cancer. MUC1 is expressed on greater than 90% of breast cancers and often elicits cellular and humoral immune responses in humans. However, these responses are not sufficiently strong to eradicate tumors. MUC1 is a candidate peptide for novel immunotherapy strategies to strongly activate the immune system to eradicate tumors expressing these epitopes. In tumors, there is strong over expression of MUC1 on tumor cells and in circulation, expression is no longer restricted to the apical domain of cells, and glycosylation is altered, revealing immunodominant tumor-specific peptide sequences.

In our preclinical studies we have utilized mice that develop spontaneous mammary gland cancer that express MUC1. MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice [1, 2]. Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) [2]. Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas in 100% of the female mice. The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously [3].

The **hypothesis** of our study is that enhancing MUC1-specific immunity will result in anti-tumor immunity. We propose to develop an optimal cancer vaccine using epithelial cell mucin MUC1 peptides or protein or MUC1-expressing tumors presented by DCs as immunogen. The most successful therapies will be tested in a phase I clinical trial. An additional hypothesis is that tolerance occurs within the tumor environment, although immunization strategies can be developed to overcome tolerance.

Our **specific aims** are: **1)** to assess the effectiveness of vaccine formulations against MUC1 in the prevention and treatment of spontaneous breast carcinomas in mice and **2)** to translate an effective vaccine strategy into a Phase I clinical trial in patients with minimal residual disease. The clinical trial protocol and the patient consent form for aim 2 are included in this annual report. Both have received FDA (IND # 12155) and Mayo IRB (IRB 782-05) approval. These documents were formally submitted on May 11, 2005, for USAMRMC approval. These approval processes are underway.

The performance period for this grant has been extended by twenty-four months, from 14 September 2005 to 14 September 2007. The Assistance Agreement is included in the appendix.

RESULTS (BODY)

Specific Aim 1: To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice.

Our preclinical studies were completed at the end of year three and reported last year. Two of the published papers describing these results are included in the appendix (those which were not included last year).

Specific Aim 2: To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden.

Description of Clinical Trial The clinical trial entitled "MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" and the Patient Consent Form are included in the appendix. The proposal for the clinical trial has been approved by the FDA **(IND#12155)** and the Mayo Institutional Review Board **(IRB 782-05)**. We have included in the appendix the approval notice from the IRB. The notice mentioned the treatment of prisoners, which is a phrase included in all approvals issued by the Mayo IRB. We have filed an amendment with the IRB stating that prisoners are not an appropriate population for this study, due to the length of follow-up. We have included in the amendment a revised list of study personnel, at the suggestion of Dr. Inese Z. Beitins, Human Subjects Protection Scientist, AMDEX, who is in charge of the review of the clinical trial prior to submission to the Department of Defense Human Subjects Research Review Board (HSRRB). A copy of the amendment is included in the appendix. The amended 1572 form listing investigators who will be involved in the study and the Investigator's Brochure have been submitted to the FDA for approval. The Investigator's Brochure is included in the appendix.

The study population will consist of patients who have had histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy and have completed "standard therapy" twelve months prior to enrollment in our clinical trial. Although we had originally proposed to use patients with high and low tumor burden, our findings regarding patients with high tumor burden suggested that this category of patient was not optimal for immunotherapy. Patients with breast cancer have T cells that showed decreased proliferation in response to T cell receptor stimulation, dendritic cells with reduced levels of co-stimulatory molecules and reduced ability to mature in response to stimulation, as well as increased PGE_2 (an immunosuppressive protein) levels in circulation [4]. These factors suggest that individuals with tumor burden would not be optimally responsive to immunotherapeutic strategies. Thus, we have chosen to test our cancer vaccine in patients free of detectable breast cancer at the time of registration. Patients must have had MUC1-positive breast cancer (90% of breast cancers are MUC1 positive) and be HLA-A2 positive. We will enroll 45 patients total at Mayo Clinic Rochester, Mayo Clinic Scottsdale, and Mayo Clinic Jacksonville. We can easily meet this criterion of patient number (see below patient population). There is a definite need to continue to test therapeutic vaccines in cancer patients and to continue to develop them. It is important to define different forms of antigen, different adjuvants, different formulations and different delivery systems [5, 6].

Patient Population

Breast cancer is the most common type of tumor seen at Mayo Clinic. Considering all three sites, a total of 1,736 new breast cancer patients were seen in 2003 including 1,084 at the Rochester campus, 228 at the Jacksonville campus and 424 at the Scottsdale campus. We have access to all the patients with breast cancer seen at all three Mayo campuses. In addition to a large clinical practice, the Mayo Clinic records system allows review of patient data going back almost one century. We also have data from breast cancer patients entered on prospective clinical trials over the last 30 years. Since 2000 we have prospectively recruited 877 women to a breast cancer patient registry in which a lifestyle and family history questionnaire is obtained. Of these 877 patients, 804 (92%) have provided a blood sample for DNA extraction and 364 patients (42%) have paraffin-embedded tissue available. We have compiled a list of patients that would be eligible for this clinical trial. Once we have completed the review process, these patients will be contacted regarding their interest in participating in the trial. Interest is very high, as immunotherapy has been shown to be a non-toxic therapy.

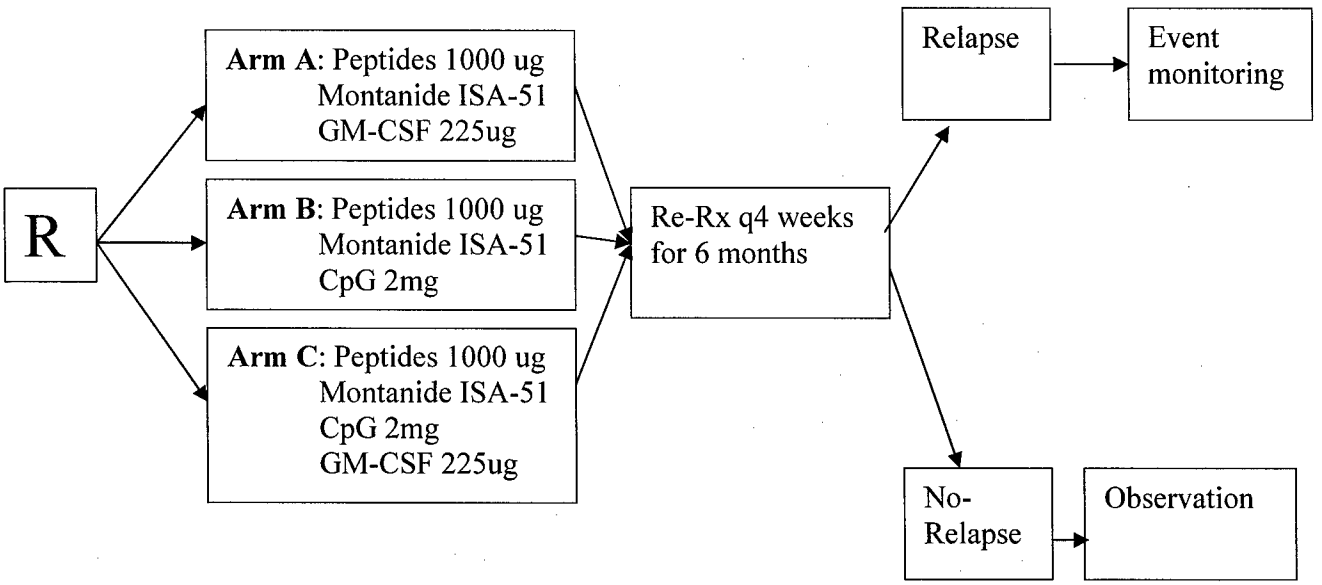
The trial will test MUC1 class I peptide (STAPPVHNV), HER-2/neu class I peptide (ILHNGAYSL) and HER-2/neu class II peptide (KVPIKWMALESIL) (1000 μg of each peptide) delivered in Montanide ISA-51 and compare GM-CSF with unmethylated CpG oligodeoxynucleotides as immune adjuvants. Few vaccines have been tested in the optimal setting of minimal residual disease. CpG unmethylated oligodeoxynucleotides are a novel adjuvant that promote strong, antigen-specific T cell responses and help to overcome immune tolderance [7, 8].

The peptides have been synthesized and vialed by Clinalfa (Merck Biosciences AG) and are being stored at the Mayo Clinic Comprehensive Cancer Center in Rochester. The peptides are certified for use in

human studies and they fully comply with the international ethical and scientific quality standards, as sated in "ICH Guideline E6: Good Clinical Practice". The "Certificate for Analysis" for each peptide is included in the appendix.

The schema for the clinical trial is shown (Fig. 1).

Figure 1



KEY RESEARCH ACCOMPLISHMENTS

- The preclinical research was completed and described in the annual report for year 3.
- Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L. and Gendler, S.J. (2004) Therapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous breast cancer model. Breast Disease 20:53-63.
- Basu, G.D., Pathangey, L.B., Tinder, T.L., LaGioia, M., Gendler, S.J., and Mukherjee, P. COX-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. Molecular Cancer Research 2:632-642.
- Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L., and Gendler, S.J. Therapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous mammary cancer model. International Society for Biological Therapy of Cancer, platform presentation in San Francisco, November 2004.

REPORTABLE OUTCOMES

- The Clinical Trial and Patient Consent are included in the appendix. They have received FDA (IND # 12155) and Mayo IRB (IRB 782-05) approval.
- These documents were submitted to the Department of Defense HSRRB on May 11, 2005.

Time Table of Protocol Development

- Clinical protocol concept approved by Mayo Clinic Cancer Center 12-11-03
- Completed Mayo Clinic Cancer Center Peer Review process 5-4-04
- List of recommendations by FDA (pre IND conference) 4-21-04
- Peptides synthesized and vialed by ClinAlfa® for use in this clinical trial:
  1. Her-2/neu (435-443) 7-16-04
  2. Her-2/neu (883-899) 8-6-04
  3. MUC1(950-958) 11-10-04
- Completion of IND documentation and submission to FDA on December 17, 2004.
- FDA approval (IND # 12155)
- Mayo IRB approval April 22, 2005 (IRB 782-05)
- Submission to DOD HSRRB on May 11, 2005
- Submission to FDA of the revised 1572 and Investigator's Brochure on September 15, 2005
- Submission to Mayo IRB of amendment, which excludes prisoners from the study population and reduces the number of personnel involved in the study (September 12, 2005)
- Upon approval by DOD HSRRB, study activation

Reprints included:

Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L. and Gendler, S.J. (2004) Terapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous breast cancer model. Breast Disease 20:53-63.

Basu, G.D., Pathangey, L.B., Tinder, T.L., LaGioia, M., Gendler, S.J., and Mukherjee, P. COX-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. Molecular Cancer Research 2:632-642.

CONCLUSIONS

All of the vaccine strategies have elicited an immune response in mice. Animals developed mature CTLs which were lytic *in vitro* against MUC1-expressing tumor cells. Lytic activity was detected without further *in vitro* stimulation. However, in most cases the spontaneous tumors progressed. The CTLs, while active outside of the environment of the tumor, were tolerized and unreactive to MUC1 (a target antigen) in the vicinity of the tumor. Tumors were found to exhibit several known escape mechanisms, such as the production of immunosuppressive factors such as Cox-2 and PGE_2 and down-modulation of MHC class I molecules on the cells. CTLs that were adoptively transferred into the tumor-bearing mice were used to follow the development of tolerance, which occurred within about three weeks following injection. Stimulation of co-stimulatory molecules, especially CD137, a member of the TNFR family, together with adoptive transfer of MUC1-specific T cells, resulted in significantly reduced tumor burden in MMT mice. The CD137-stimulated CTLs appeared to remain lytic against the tumor in the tumor environment and tolerance/anergy was alleviated. CTLs (tumor infiltrating lymphocytes) recovered from the tumors were lytic against MUC1-expressing tumor cells (MMT tumor cells in culture, as well as B16/MUC1 cells) and they proliferated in response to DCs presenting MUC1. A peptide vaccine was tested in MUC1.Tg mice using MUC1-expressing tumors injected subcutaneously. Two class I-binding peptides from MUC1 (APGSTAPPA and SAPDTRPA from the MUC1 tandem repeat region) and a class II helper peptide (TPPAYRPPNAPIL) were used together with CpG ODN and GM-CSF in Incomplete Freund's Adjuvant. The peptide vaccine completely inhibited the growth of MUC1-expressing tumor cells and elicited a strong immune response. This strategy will be utilized in the clinical trial described in Aim 2.

We showed reduced functionality of T cells and dendritic cells in breast cancer patients with large tumor burden. Ours was the first study to evaluate DC and T cell function from the same breast cancer patients and from age-matched controls. We chose to limit the study population to patients with resected stage II or III breast cancer who completed "standard therapy" 12 months previously and have no evidence of disease. This time lapse should allow the immune system to recover to normal levels prior to the first immunization. The clinical trial, which is included in the appendix, has received FDA 9IND #12155) amd ORB (782-05) approval. GMP-grade peptide synthesis has been completed for all three peptides (see appendix). Submission to the DOD Human Subjects Research Review Board occurred May 11, 2005. We anticipate that the trial will open in late 2005. We presently have a list of patients who would be eligible for this trial. We anticipate that there will be rapid accrual.

Future Studies

The clinical trial will open shortly. We will enroll the patients and perform the immunologic assays once final USAMRMC approval has been received.

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APPENDICES

Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L. and Gendler, S.J. (2004) Therapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous breast cancer model. Breast Disease 20;53-63.

Basu, G.D., Pathangey, L.B., Tinder, T.L., LaGioia, M., Gendler, S.J., and Mukherjee, P. (2004) COX-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. Molecular Cancer Research 2;632-642.

Clinical Trial MC0338 – MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas

Patient Consent Form

IRB Approval of MC0388 and Patient Consent April 22, 2005 (IRB 782-05)

Amendment for IRB 782-05 Submitted 9/12/05

Investigator's Brochure (IB): to be submitted September 15, 2005

Submission of Revised Form 1572 (Statement of Investigator) to FDA: to be submitted September 15, 2005

IRB approval notice for IB July 20, 2005

BB-IND#: 12155 December 21, 2004 letter

Clinalfa Standards for Human Studies (MERCK)

Certificate of Analysis for HER 2/neu (435-443)

Certificate of Analysis for HER 2/neu (883-899)

Certificate of Analysis for MUC1 (578-586)

Copy of Assistance Agreement, which extends the grant from 15 August 2001 to 14 September 2007 (research ends 14 August 2007)

Breast Disease 20 (2004) 53–63
IOS Press

Therapeutic Efficacy of MUC1-Specific Cytotoxic T Lymphocytes and CD137 Co-Stimulation in a Spontaneous Breast Cancer Model

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Abstract. To study immunology in breast tumors, we have utilized a mammary gland adenocarcinoma model in which mice develop spontaneous tumors of the mammary gland which are initiated at puberty and express a human tumor antigen, MUC1. MUC1 (CD227) is over-expressed in 90% of human breast cancers and its glycosylation status and pattern of expression in cancer cells is altered. Humoral and cellular responses to MUC1 have been reported in breast cancer patients and therefore, MUC1 is being evaluated as a target for immune intervention. This mouse model of spontaneous breast cancer allows the evaluation of anti-MUC1 immune responses at all stages of the disease. In this report, we review the model as it pertains to a) the development of the tumor, b) MUC1 expression, and the native immune responses against MUC1 as tumors progress, and c) the immune suppressive microenvironment within the developing tumor. Finally, we report our latest findings describing the therapeutic efficacy of adoptively transferred MUC1-specific cytotoxic T lymphocytes (MUC1-CTL) in these mice and discuss ways to increase their effectiveness by agonistic monoclonal antibody against CD137 T cell costimulatory molecule.

INTRODUCTION

Breast cancer remains a major health problem, accounting for approximately 40,000 deaths each year in the United States. Interest is high in developing non-toxic therapeutic approaches to complement toxic surgical and chemotherapeutic strategies. The recent molecular identification of tumor antigens recognized by cytotoxic T cells (CTLs) derived from cancer patients has initiated a new era in tumor immunology. MUC1 (CD227) is a cell-associated mucin that is developmentally regulated and aberrantly expressed by more than ninety percent of breast carcinomas [1–3]. The recent description of MUC1 as a target for CTLs has raised interest in using this protein as a target for immunotherapy.

MUC1 is a transmembrane protein that exists as a large extended rod protruding from the cell membrane into the lumen of ducts and glands. The core protein consists mainly of a twenty amino acid sequence repeated from 30–90 times. These tandem repeats (TR) serve as the scaffold for O-linked oligosaccharides that cover the polypeptide core. In cancer, MUC1 expression is greatly increased on cells and in circulation, its expression is no longer restricted to the apical cell surface, and its glycosylation is altered. Both cellular and humoral immune responses to MUC1 have been reported (reviewed in [4]). However, these responses

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are not sufficiently strong to stimulate CTL killing, as most breast adenocarcinomas express MUC1 and these tumors still progress. Thus, there is a need for studies to devise effective presentation of MUC1 immunogens to stimulate immune cells to kill tumor cells. The mouse has not been a suitable preclinical model for testing vaccines, as human MUC1 differs in sequence from mouse Muc1 and is a foreign antigen in the mouse. (Human MUC1 is designated MUC1, mouse as Muc1.) We have developed MUC1-expressing mice that spontaneously develop mammary gland tumors for use in pre-clinical studies. This model effectively mimics the human situation and provides a powerful system in which to study tolerance and inactivation of CTLs in the tumor microenvironment.

Bitransgenic Mice Develop Spontaneous Mammary Gland Cancer Accompanied by Lung and Bone Marrow Metastases

Human MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice [5–7]. MUC1.Tg mice carry the full-length human MUC1 gene driven by its own promoter; they express normal levels of MUC1 in a tissue specific manner and are therefore not a model of MUC1 over expression [8]. These mice exhibit T and B cell tolerance to the MUC1 antigen, thus providing an excellent model system where MUC1-specific therapy can be studied in the context of immune tolerance [5,9]. In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) [10]. Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Hyperplastic alveolar nodules (HANs) can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 49 days onwards (a schematic representing the model and the tumor progression is shown in Fig. 1). Tumor progression is quite rapid, reaching 10% of body weight by about 24 weeks [6]. 100% of the female MMT mice get tumors. Tumors arise with synchronous kinetics

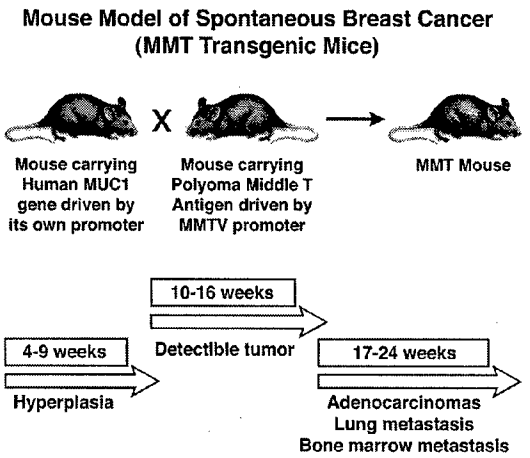

Fig. 1. Schematic representation of a mouse model of spontaneous metastatic breast cancer and approximate time-line of tumor progression from hyperplasia to adenocarcinomas and metastasis.

and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast [11]. Lung and bone marrow metastases were detected in MMT mice by 4 months of age [6]. Bone marrow metastasis was determined by staining bone marrow cells from MMT mice with pan-cytokeratin and MUC1, markers commonly used to detect epithelial tumor cells. About four percent of bone marrow cells were positive for both pan-cytokeratin and MUC1 by flow cytometry [6]. It is important to note that mortality in human breast cancer patients is directly associated with lung and bone metastasis. The MMT mouse is, therefore, an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously.

MUC1 Expression Increases as MMT Tumors Progress

Tumor sections from 6, 12 and 20 week old non-immunized MMT mice show strong heterogeneous expression of MUC1 as tumors progress (Fig. 2). As compared to normal mammary gland, tumors express higher levels of MUC1 with increase in expression as tumors progressed from 6 weeks to 18 weeks (Fig. 2). MUC1 staining was determined using two antibodies, one recognizing the cytoplasmic tail of MUC1 (CT2) and the other recognizing the tandem repeat (TR) epitope of MUC1 (B27.29). CT2 antibody recognizes both mouse and human cytoplasmic tail (CT) of MUC1. It

is a monoclonal antibody raised in Armenian hamster. B27.29 is a mouse monoclonal reactive only against human MUC1 [12,13]. The staining pattern for B27.29 and CT2 was similar, hence, only B27.29 is shown in Fig. 2. MUC1 was expressed throughout the cytoplasm and around the cells in a pattern similar to that observed in human breast carcinomas. Thus, mammary gland tumors that occurred spontaneously in the MMT mice over-expressed the transgene protein, MUC1, in a manner similar to humans.

Cellular and humoral responses are not evidenced in MMT mice as tumors progress

In some breast cancer patients, humoral and cellular immune responses against MUC1 have been demonstrated. Thus, we determined native cellular and humoral immune responses in the non-immunized MMT mice as tumors progressed. At different stages of the tumor (6, 14, and 21 weeks), T cells were examined for expression of early and late activation markers (CD69 and CD25), as well as for expression of intracellular cytokines including IFN-γ, IL-2, IL-4, IL-5, and IL-10 in response to MUC1. No significant anti-MUC1 immune responses were evident in MMT mice as tumors progressed. Furthermore, we were unable to detect presence of T cells recognizing H-2D^b/MUC1 tetramer nor did we detect precursor cytotoxic T lymphocytes (CTLs) against MUC1 at any stage during tumor progression. Dendritic cell (DC) maturation status as determined by B7 co-stimulatory receptor expression and IL-12 production also remained unchanged as the tumor progressed. With regards to humoral immune response, circulating antibody to MUC1 was undetectable by specific ELISA at any time during tumor progression. These data taken together clearly indicate that naturally occurring cellular or humoral immune responses in non-immunized MMT mice were non-detectable [6]. This lack of detectable anti-MUC1 immune response in the MMT mice implies that the immune tolerance to MUC1 is not broken by aberrantly expressed tumor-specific MUC1. In general, immune tolerance to a particular self-antigen can be broken if the immune cells encounter large amounts of the antigen systemically. In some human breast cancers, a low level anti-MUC1 immune response is generally associated with high levels of shed MUC1 in the serum which is not the case in MMT mice.

Increased Levels of MUC1 in the Serum Determine if MUC1-Specific Immune Responses will be Elicited

As tumors progressed in the MMT mice, MUC1 serum levels increased only slightly as compared to age matched MUC1.Tg mice with maximum reaching to 1500–2500 Units/ml of serum at 24 weeks of age. In age matched female MUC1.Tg mice, serum MUC1 levels ranged from 500 to 1200 Units/ml, which is likely to depend upon their estrous cycle status. The low levels of circulating MUC1 may explain the lack of an immune response to MUC1 in non-immunized MMT mice. When MMT mice were immunized with liposomal MUC1-TR and human recombinant interleukin-2, we observed significant increase in the levels of serum MUC1 as compared to untreated MMT mice. This increase in serum MUC1 directly corresponded to the increased CTL activity in these mice [6]. These results suggested that high levels of circulating tumor antigen, MUC1, may activate MUC1-specific CTL that are capable of specifically lysing MUC1-expressing tumor cells *in vitro*. We also detected low levels of circulating antibodies to MUC1 in the immunized mice suggesting that the high level of circulating tumor-associated MUC1 has changed the antigenic profile and elicited a moderate level of humoral response to MUC1. Antibodies reactive with MUC1 have been reported in a small percent of breast cancer patients [14,15]. Although the effectiveness of a humoral immune response against solid tumors is not established, it once again parallels that observed in humans. Taken together, these data demonstrate that as observed in humans, immunization strategies elicited MUC1-specific CTLs which were unable to kill the spontaneously arising breast tumors. These CTLs were found to kill MUC1$^+$ tumor cells *in vitro*, secrete IFN-γ, and express perforin and granzyme B. Despite the presence of mature functional CTLs, these mice grew tumors [6,16]. We therefore postulated that the growing MMT tumor cells evaded immune recognition and killing, a phenomenon that is becoming increasingly critical to consider in designing future immune-based therapies.

Characteristics of the MUC1-Specific CTLs

To test whether MUC1-specific CTLs enter the mammary tumor bed and are active within the tumor microenvironment, we adoptively transferred the cytolytically active MUC1-specific CTL clone into MMT mice [17,18]. These MUC1-specific CTL clone were generated from a CTL line that was originally derived

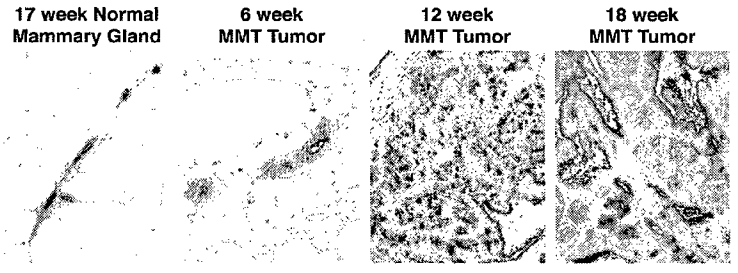

Fig. 2. MUC1 expression increases as tumors develop in MMT mice. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6, 12 and 18 week old MMT mice were stained with B27.29, a monoclonal antibody reactive with MUC1 TR. B27.29 antibody is directly conjugated to horse radish peroxidase (HRP). specific staining was observed on lumenal surface of mammary epithelial cells and staining pattern is similar to that seen in humans. Increase in MUC1 expression is observed as tumors developed from 6 weeks to 18 weeks. As control, normal mammary gland from 17-week old MUC1.Tg mice was used. MUC1 expression is restricted to the apical surface in normal glands as compared to tumors and the staining is less intense than in MMT tumors. Images were captured at 200X magnification.

from a MUC1-expressing pancreatic cancer mouse model that develops spontaneous tumors of the pancreas [17]. Unlike the MMT model, these mice naturally developed MUC1-specific CTLs as the pancreatic tumor progressed. The MUC1-specific CTL lines are CD8+ T cells that recognize several of the MUC1-derived peptides (Fig. 3(A)). Fourteen different MUC1 TR 9 mer peptide sequences and one MUC1 cytoplasmic tail (CT) 17 mer peptide were used to determine epitope recognition. Dendritic cells were pulsed with these peptides prior to use as targets for the CTL line and CTL clone. The line recognizes several of the MHC class I-restricted MUC1 TR peptides and the CT peptide as illustrated in Fig. 3(A). Thus, the CTL line is comprised of a heterogeneous population of T cells, expressing predominantly T cell receptor (TCR) Vβ5, 11, 13 and 2 and V*a*8, 3, 11, and 2. The CTL clone, on the other hand, recognizes only a H-2D^b MHC class I-restricted immunodominant epitope of MUC1 (APGSTAPPA) and expresses Vβ5 and Vα2 [18]. When CTL line was adoptively transferred (intravenously) into MUC1.Tg mice prior to challenge with either the B16 melanoma cells expressing MUC1 transgene (B16.MUC1), or the C57 mammary tumor cells expressing MUC1 (C57mg.MUC1), the mice were protected against both types of tumor cell challenge and furthermore, developed memory T cells that could be transferred to protect naive MUC1.Tg mice from further tumor challenge [17,18]. These MUC1-specifc CTL clones expressed high levels of perforin and granzyme B as determined by confocal microscopy and flow cytometry. Perforin and granzyme B are cellular components of cytolytic granule of CTLs and NK cells that mediate lymphocyte-dependent killing and low expression of these proteins on CTLs suggest weak cytotoxicity [19,20]. Figure 3(B) shows a representative flow cytometric dot plot of CTLs stained with anti-granzyme B antibody that was directly conjugated to phycoerythrin (PE). Almost 100% of the cells express high levels of intracellular granzyme B (Fig. 3(B)). Similarly, CTLs stained with a specific perforin antibody showed high intensity of intracellular staining by confocal microscopy (Fig. 3(B)), thus, confirming the high cytolytic activity of the MUC1-specifc CTLs.

MUC1-Specific CTLs Delay Tumor Growth in MMT Mice

To test the efficacy of the MUC1-specific CTL in MMT mice, we determined if the CTLs could enter the mammary tumor bed and whether they remained active within the tumor micro-environment. We adoptively transferred cytolytically active MUC1-specific CTL clones into 14-week-old MMT mice, which had palpable tumors. To determine if the infused CTLs could home to the tumor site, we labeled the CTL clone with carboxy-fluoresceinsuccinimidyl ester (CFSE, an *in vivo* tracking dye) prior to adoptive transfer of 1 x 10^7 CTLs intravenously. We observed that CFSE-labeled CTL home to the tumor site and lymph node by 2 days post infusion and undergo proliferation as evidenced by the discrete population of cells with distinct CFSE staining intensities. CTLs were detected up to 20 days post infusion (Fig. 4). Few to no CTLs were detected within the thymus or the spleen. To determine if the infused CTLs remained active within the tumor environment, we infused 3 week-old MMT mice with unlabeled 1 x 10^7 CTLs intravenously and boosted every 3 weeks with the same. Final infusion was given at 15 weeks and at 17 weeks of age, the mammary tumors were removed, tumor-infiltrated lymphocytes (TILs) were isolated and sorted for the infused CD8+ CTL

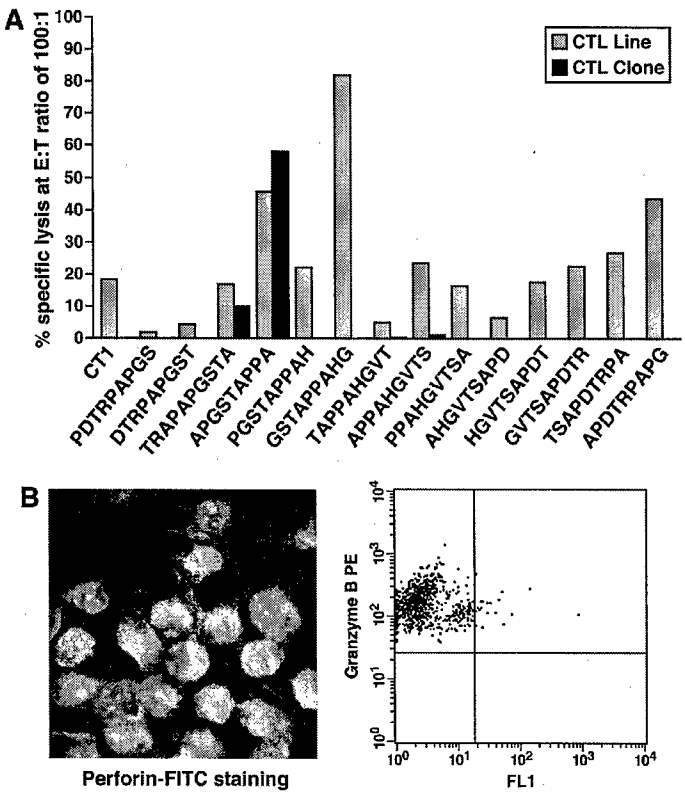

Fig. 3. A. Epitopes recognized by MUC1-specific CTL line and clone. Fourteen different MUC1 TR 9 mer peptide sequences and a MUC1 cytoplasmic tail 17 mer peptide were used to determine epitope recognition. Dendritic cells were pulsed with the peptides at 10(-6)M concentration prior to use as targets for the CTL line and CTL clone. A standard 8hr ^{51}Cr-release assay was performed. Specific lysis was performed with several effector to target (E : T) ratios. Figure shows data from 100:1 effector to target ratio. Recognition of the peptides by CTLs is gauged by the percent of specific lysis. Lysis at and above 18-20% is thought to be significant. Thus, MUC1 line consists of several CTL populations that recognize eight different TR peptides and the CT peptide. MUC1 clone recognizes only APGSTAPPA peptide. Degree of lysis directly correlates to the affinity by which the peptide is recognized by the CTL. CTLs seem to recognize APGSTAPP Aand GSTAPPAHG with the highest affinity. B.MUC1-specific CTLs express high levels of perforin and granzyme B. CTLs were permeabilized using the Pharmingen Permeabilization kit and stained with either perforin-FITC antibody (Alexis Biochemicals, San Diego, CA) and examined by confocal microscopy (left panel); or with granzyme B antibody directly conjugated to PE (Caltag Laboratories, Burlington, CA) and analyzed by flow cytometry (right panel).

clone based on their CD8 expression and TCR Vβ5 expression (Fig. 5(A),2nd panel). TILs isolated from age matched MMT mice that were not infused with CTLs were used as controls (Fig. 5(A), 4th panel). The CTL clone maintained *in vitro* with the same profile as the sorted TILs were used as positive control (Fig. 5(A), 1st panel). The sorted cells were used in three assays to determine: 1) if these cells were hypo-responsive to MUC1 and therefore were tolerant to MUC1 antigen, 2) if these cells maintained their cytotoxicity after encounter with the tumor cells *in vivo*, and 3) if the sorted CTLs expressed granzyme B, a protein needed for the CTLs to be functionally lytic. In Fig. 5(B), we clearly show that in contrast to the CTL clone, the sorted Vβ5$^+$ /CD8$^+$ TILs had significantly reduced proliferation in response to MUC1 presented on irradiated DCs, suggesting that the infused CTLs become tolerized to tumor antigen MUC1 after encounter with the growing mammary tumor cells. Similarly, the sorted Vβ5$^+$ /CD8$^+$ TILs were unable to kill tumor cells expressing MUC1 *in vitro*, while the same cells that had not encountered tumor cells and were maintained in tissue culture remained highly cytolytic (Fig. 5(C)), suggesting that the infused CTL became cytolytically nonfunctional in the tumor environment. This cytolytic capability of the sorted TILs was further confirmed by the observation that the sorted Vβ5$^+$/CD8$^+$ TILs from the tumors showed significantly lower levels of granzyme

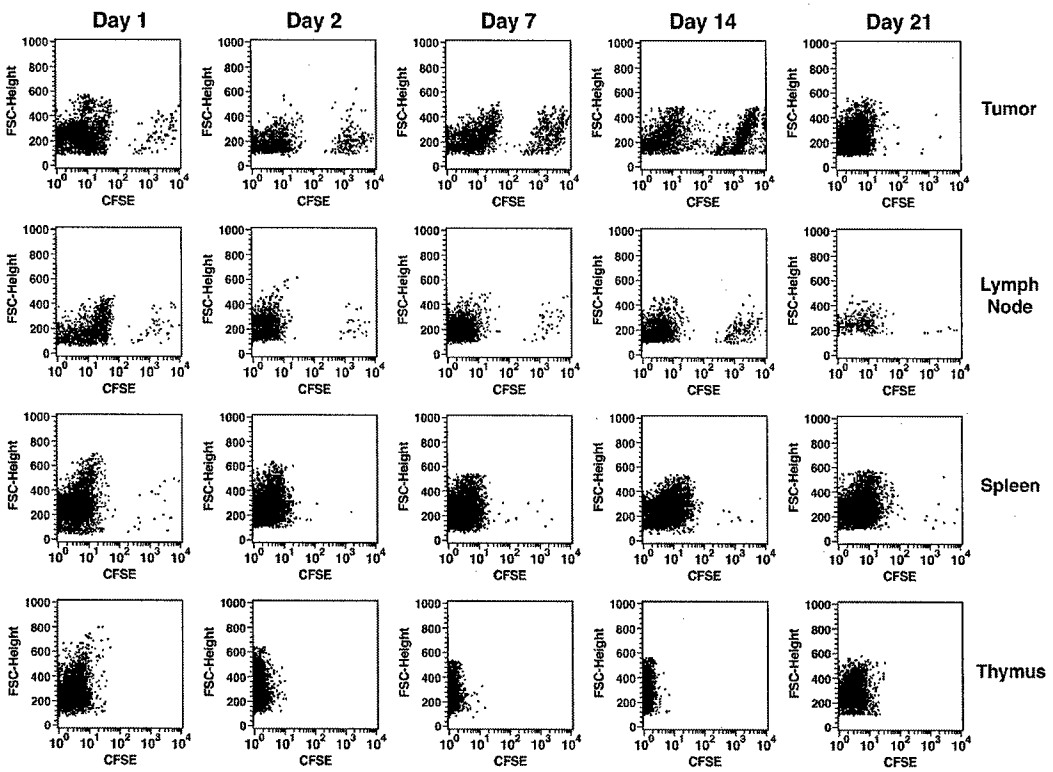

Fig. 4. CFSE labeled MUC1-specific CTL home to the lymph node and mammary gland tumor. CTL clone were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were incubated with 2 μl of 5 mM CFSE per 1×10^7 cells/ml in PBS-0.1% FBS for 10 minutes at 37°C and quenched by adding 5 times the volume of ice-cold media. 2×10^7 CFSE-stained CTLs were injected intravenously into 14-week old tumor bearing MMT mice. At various times post CTL infusion, TILs, lymph node cells, spleen cells and thymic cells were isolated and monitored for CFSE staining by flow cytometry. $n = 5$ mice and a representative dot plot is shown. By 21 days post infusion, CFSE labeled cells were undetectable.

B staining as compared to the CTLs that were maintained in culture (Fig. 5(D)). These findings are extremely significant, as immunotherapy can now be designed to reverse this established tolerance or prevent the induction of tolerance. The treatment group that includes CTL + CD137 monoclonal antibody (mAb) is represented in this Figure, but the experiment is described in a later section.

Nevertheless, when CTLs were continuously infused in MMT mice starting at 3 weeks of age, we observed a significant reduction in tumor growth and progression (Fig. 6). Clearly, adoptive transfer of MUC1-specific CTL early during tumorigenesis can reduce tumor burden and enhance survival in these mice. However, once CTL infusions were stopped, the tumors grew back. These results suggest strongly that immunotherapy can work if the tumor microenvironment is somehow altered. For example, if mediators of tumor-induced immune-suppression can be reduced, co-stimulation for CTL function can be provided to overcome the tolerizing effects of the tumor microenvironment, and most importantly, if tumor-cell proliferation can be restricted.

Immunosuppressive Tumor Microenvironment in the Mammary Gland Tumor

Since infused CTLs became tolerant and cytolytically non-functional within the tumor microenvironment, we evaluated the presence of immunosuppressive mediators in mammary tumors. Data revealed that the tumor cells secrete IL-10 and TGF-β that are partly responsible for the down-regulation of CTL activity [6]. IL-12 production and expression of co-stimulatory receptors by DCs was also found to be reduced, suggesting sub-optimal antigen presentation within the tumor micro-environment. In addition, tumor cells down-regulated surface major histocompati-

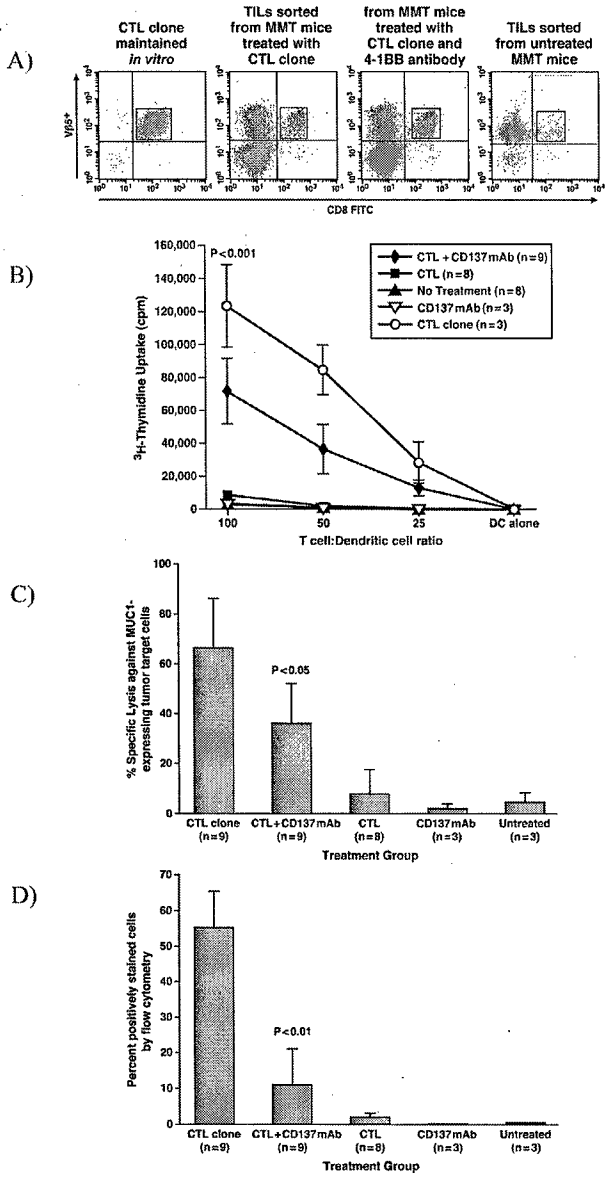

Fig. 5. Adoptively transferred CTL become tolerant to MUC1 antigen and are cytolytically inactive within the tumor microenvironment. TILs were isolated from tumors of MMT mice that received adoptively transferred MUC1-specific CTL clone ($V\beta5^+/CD8^+$ T cells). CD137 mAb was injected every week at 100 ng/mouse/100 µl intraperitoneally. A) Flow cytometric profile of TCR $V\beta5^+/CD8^+$ T cells sorted from TiLs by flow cytometry (box represents the population that was sorted). Profile of untreated MMT and MMT treated with CD137 mAb alone looked identical and therefore we chose to show only one group. B) Proliferation by 3H-thymidine uptake of the sorted T cells. Proliferation was determined in response to 25 mer MUC1 peptide (STAPPAHGVTSAPDTRPAPGSTAPP) presented on dendritic cells. C) Cytotoxic activity by 51Cr-release assay of sorted T cells against MUC1-expressing B16.MUC1 melanoma tumor cells. As positive controls, CTL clone maintained *in vitro* was used and as negative controls TCR $V\beta5^+/CD8^+$T cells from mammary tumor of age matched untreated MMT mice was used. D) Granzyme B staining of sorted T cells by flow cytometry. Cells were permeabilized and antibody to Granzyme B directly conjugated to PE from B.D. Pharmingen was used at 1 ug/10^6 cells to determine intracellular staining using flow cytometry. Treatment groups include: MMT treated with CTL; MMT treated with CTL + CD137 mAb; MMT treated with CD137 mAb; and untreated MMT. p-values are shown in the figure and represent significant differences between CTL + CD137 group as compared to untreated or α–CD137 alone or CTL alone groups. 6 mice were enrolled in the α–CD137, however, 3 mice were found morbid prior to end of experiment and were removed from the study.

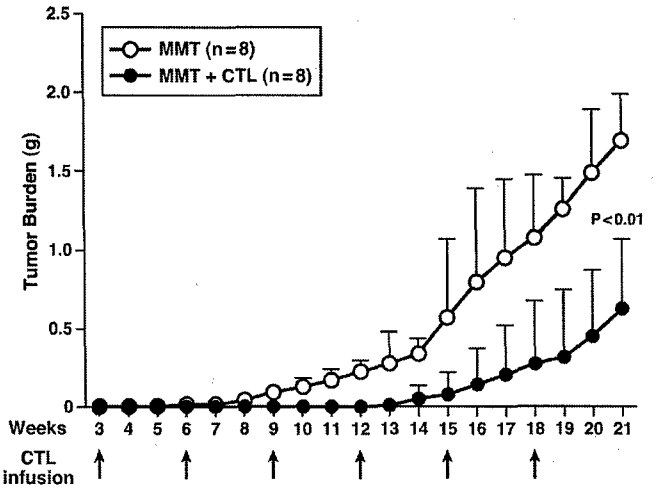

Fig. 6. Adoptively transferred MUC1-specific CTL clone inhibits tumor progression in MMT mice. MUC1-specific CTLs (2×10^7 cells i.v.) were infused into MMT mice starting at three weeks of age. Infusions were given every three weeks from week 3 onward. Six infusions were performed. Tumor growth was inhibited until the cessation of CTL injections, at which time tumors began to progress. CTL infused MMT mice showed significantly lower tumor burden as compared to untreated MMT mice ($p < 0.01$).

bility complex (MHC) class I molecules to avoid immune recognition as well as expressed higher levels of $CD4^+CD25^+$ T regulatory cells [6,16]. Recently, we have found over expression of COX-2 in the MMT tumors by western blot analysis (Fig. 7) as well as by immunohistochemistry (data not shown). COX-2 is an inducible enzyme that is over expressed in many tumors and is involved in many aspects of tumorigenesis. COX-2 converts arachidonic acid to prostaglandins, especially prostaglandin E2 (PGE2), a well-characterized immune cell suppressor [21–24]. In MMT mice, we found significantly higher levels of serum PGE2 as compared to normal mouse sera, suggesting that the COX-2/PGE2 pathway may be partly responsible for the immune suppressive tumor micro-environment in MMT mice. We have recently shown that T cell and DC functions in newly diagnosed breast cancer patients are impaired and that over expression of COX-2 and PGE2 may play a significant role in inducing such suppression [25]. Together, these data indicate that mammary gland tumor cells utilize a variety of immune evasion mechanisms to avoid CTL killing. Managing all of these immune evasion pathways seems formidable. On one hand, we have immune responses against the tumor and, on the other hand, there is tumor-induced immune suppression. Tilting the balance towards more sustained and increased CTL activity may be easier to accomplish than addressing every one of the tumor evasion mechanisms. One way to accomplish this is by providing the activated CTLs with appropriate co-stimulation to increase their efficiency in killing tumor cells.

CD137 mAb can Reverse Tolerance in vivo in MMT Mice and has a Synergistic Anti-Tumor Effect when Combined with MUC1-Specific CTL Therapy

The CD137 glycoprotein is a member of the tumor necrosis factor receptor superfamily expressed on primed but not on naïve $CD4^+$ and $CD8^+$ T cells. CD137 binds to a specific ligand (CD137L) expressed on several antigen presenting cells (APCs) and signals either through ligand binding or by specific agonistic antibody to deliver a dual mitogenic signal for further T cell activation and proliferation. It has been shown that administration of CD137 mAb can amplify T cell-mediated immune responses and can eradicate established tumors [26,27]. Recent experiments suggest that anti-tumor effect of CD137 antibody is to reverse T cell tolerance/anergy [28] that is so often induced by tumor cells. Thus, we evaluated the efficacy of CD137 antibody therapy in reversing tolerance in our *in vivo* breast cancer model, since we have already established that adoptively transferred CTLs become tolerized to MUC1 and are cytolytically non-functional within the tumor microenvironment. We were therefore able to specifically answer whether treatment with CD137 antibody could reverse this tolerizing effect within the mammary gland tumor. MMT mice were infused with 1×10^7 CTLs starting at 3 weeks of age and boosted

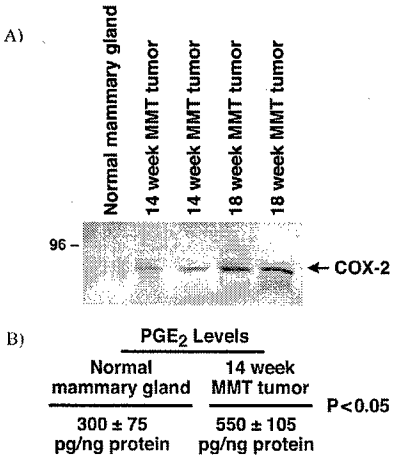

Fig. 7. MMT tumors express COX-2 and the downstream product PGE2. A) Western blot analysis of mammary tumor lysate derived from 14 and 18-week old MMT mice compared to normal mammary gland lysate. SDS-PAGE electrophoresis was performed using 12% resolving gel. 50 μgs of protein was loaded per lane. Gels were immunoblotted and probed for COX-2 with specific COX-2 monoclonal antibody (goat polyclonal, clone C20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200 dilution. MMT tumors over express COX-2 as tumors progress. B) PGE2 levels determined by specific ELISA in 14-week old MMT tumor lysate as compared to normal mammary gland lysate (n = average of 6 mice). Levels in the lysates were determined using a specific ELISA kit for PGE2 (Cayman Pharmaceuticals, Ann Arbor, MI). Manufacturer's recommended protocols were followed. Compared to normal mammary gland, PGE2 in tumor is significantly higher ($p < 0.05$).

every 3 weeks with the same. Final infusion was given at 15 weeks and TILs were sorted for $CD8^+V\beta5^+$ T cells at 17 weeks (Fig. 5(A), 3rd panel). Along with the CTL infusions, these mice received CD137 mAb at 100 ug/mouse intraperitoneally every week until mice were sacrificed at 17 weeks of age. Data clearly indicate that CD137 mAb treatment was extremely effective in reversing tolerance such that the CTL clone regains responsiveness to MUC1 antigen post CD137 treatment (Fig. 5(B)). Treatment with this antibody was also effective in restoring the cytolytic activity of the CTL clone as measured by the ^{51}Cr-release assay and granzyme B staining (Figs 5(C) and (D)). Most importantly, this reversal of CTL tolerance *in vivo* translated well to anti-tumor response. When mice were sacrificed at 17 weeks, tumor burden was evaluated. MMT mice treated with CTL alone had a tumor burden of 0.5 g that was significantly lower than untreated control mice with 0.9 g tumor ($p < 0.05$) or mice treated with CD137 mAb alone with 1 g tumor. Treatment with CD137 increased the effectiveness of CTLs since MMT mice treated with CTL and CD137 mAb had a tumor burden of 0.2 g ($p < 0.01$) (Fig. 8). The unresponsiveness of MMT tumors to CD137 mAb alone can be explained by the fact that CD137 is only expressed on activated T cells and does not work on naïve, non-primed T cells [29] and untreated MMT mice have no detectable primed T cells. Although 6 mice were enrolled in the anti-CD137 treatment arm, 3 mice were found morbid prior to end of experiment and were removed from the study. These results demonstrate that MUC1-specific CTLs are very effective against spontaneously arising MUC1 expressing breast tumors and that their efficacy can be increased and maintained for longer time with appropriate co-stimulation such as with anti-CD137 antibody. Lung metastasis was not evaluated in these mice, since tumor burden at 17 weeks even in the untreated mice is not large enough for lung metastasis to develop. The next set of experiments will determine the effect such a treatment on lung and bone marrow metastasis.

CONCLUSIONS

We have shown that the spontaneous mammary gland tumors that arise in the transgenic MMT mice appropriately models the human metastatic breast cancer. Several features of the tumor resemble the human disease, for example, the tumors arise in an immune competent host within the context of MUC1-tolerence; the tumors aberrantly over express MUC1, the tumors metastasize to the lungs and the bone marrow, and very little to no immune response against the tumor antigen, MUC1, is evident in the model. As observed in humans, treatment with anti-MUC1 immunotherapy leads to a robust cellular immune response accompanied with a moderate humoral immune response against the immunizing antigen, MUC1, which does not translate efficiently to a clinical response. We further show that the inefficiency of MUC1-specific CTLs to affect tumor burden and survival is partly due to the immunosuppressive tumor microenvironment that renders the infiltrating CTLs inactive with regards to antigen recognition and killing. Similar to observations in human breast cancer, several immunosuppressive factors were identified in the MMT tumor microenvironment. In fact, in human disease, immunosuppression has not only been described within the tumor microenvironment but also systemically. Finally, we show in our model, that the most effective anti-tumor response was generated when mice were treated with continuous intravenous infusions of MUC1-specific CTLs and that the CTL

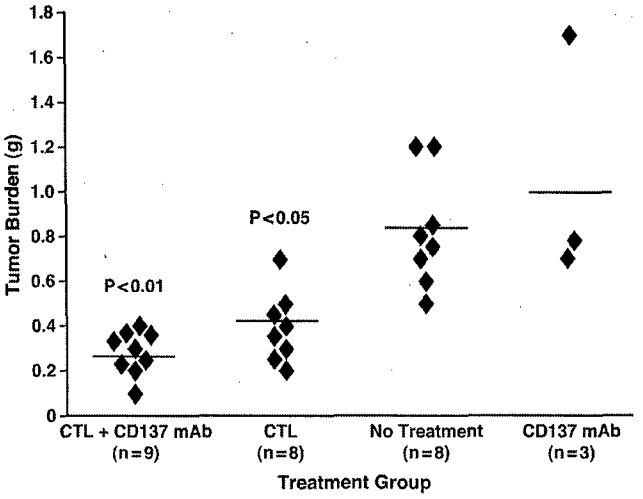

Fig. 8. CD137 mAb in combination with MUC1-specific CTL therapy is more efficient in reducing tumor burden than CTL therapy alone. MMT mice were injected with MUC1-specific CTLs (2×10^7 cells i.v.) every three weeks starting at 3 weeks: and CD137 mAb was injected at 100 ug/mice (i.p.) every week starting at 6 weeks. Individual animal data are shown as scatter plot. P values indicate significant differences between treatment groups and untreated control mice. Tumor burden was significantly lower in CTL + CD137 mAb treated mice ($p < 0.01$) and in CTL treated mice ($p < 0.05$) as compared to untreated and CD137 mAb treated mice. 6 mice were enrolled in the CD137 mAb group; however, 3 mice were found morbid prior to end of experiment and were removed from the study. CD137 mAb, clone 2A, was raised in rats against a fusion protein consisting of the extracellular domain of murine CD137 and the human immunoglobulin C region [26].

efficiency was significantly enhanced when treatment was combined with CD137 co-stimulation. From our data, we suggest that one of the mechanisms by which CD137 co-stimulation increases CTL activity is by reversing tumor-induced CTL tolerance and hence inactivity *in vivo*. These results allow us to propose that such a therapy may be beneficial for the treatment of metastatic breast cancer in humans.

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Cyclooxygenase-2 Inhibitor Induces Apoptosis in Breast Cancer Cells in an *In vivo* Model of Spontaneous Metastatic Breast Cancer

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Abstract

Cyclooxygenase-2 (COX-2) inhibitors are rapidly emerging as a new generation of therapeutic drug in combination with chemotherapy or radiation therapy for the treatment of cancer. The mechanisms underlying its antitumor effects are not fully understood and more thorough preclinical trials are needed to determine if COX-2 inhibition represents a useful approach for prevention and/or treatment of breast cancer. The purpose of this study was to evaluate the growth inhibitory mechanism of a highly selective COX-2 inhibitor, celecoxib, in an *in vivo* oncogenic mouse model of spontaneous breast cancer that resembles human disease. The oncogenic mice carry the polyoma middle T antigen driven by the mouse mammary tumor virus promoter and develop primary adenocarcinomas of the breast. Results show that oral administration of celecoxib caused significant reduction in mammary tumor burden associated with increased tumor cell apoptosis and decreased proliferation *in vivo*. *In vivo* apoptosis correlated with significant decrease in activation of protein kinase B/Akt, a cell survival signaling kinase, with increased expression of the proapoptotic protein Bax and decreased expression of the antiapoptotic protein Bcl-2. In addition, celecoxib treatment reduced levels of proangiogenic factor (vascular endothelial growth factor), suggesting a role of celecoxib in suppression of angiogenesis in this model. Results from these preclinical studies will form the basis for assessing the feasibility of celecoxib therapy alone or in combination with conventional therapies for treatment and/or prevention of breast cancer.
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Introduction

In the United States, breast cancer is the second most common cancer and contributes to 40,000 deaths in a year. If confined within the breast, the tumor can be surgically removed with an increased survival rate. However, primary tumors that metastasize to distant sites such as lymph nodes, lungs, liver, and brain correlate with poor prognosis. Complications from metastatic disease are the leading cause of cancer-related deaths. Mean survival for patients with metastatic breast cancer is 18 to 24 months. Response to chemotherapy or endocrine therapy in metastatic breast cancer patients is ~50% (1). Clearly, a need for development of novel therapies to enhance the existing triad of surgery, radiation, and chemotherapy is evident. Cyclooxygenase-2 (COX-2), the inducible form of the COX enzymes, catalyzes conversion of arachidonic acid to prostaglandin H_2, which is further converted to several other prostaglandins with diversified functions. Deregulation of COX-2 activity and downstream prostaglandins plays a vital role in carcinogenesis, inflammation, and tissue damage (2-5). COX-2 is overexpressed in many cancers including breast cancer, and the major functional prostaglandin in breast cancer is prostaglandin E_2 (PGE_2). Overexpression of COX-2 protein and PGE_2 during carcinogenesis is implicated in proliferation, invasion, apoptosis, immune suppression, and angiogenesis. COX-2 is induced by a variety of factors including tumor promoters, cytokines, growth factors, and hypoxia. Importantly, selective inhibition of this enzyme reduces adenocarcinoma formation and cancer progression in preclinical animal models (6-8). The first direct evidence of COX-2 function in cancers came from the study by Eberhart et al. (9), documenting significant elevations in COX-2 expression in 85% of human colorectal carcinomas and 50% of colorectal adenomas. COX-2 overexpression has since been found in many other human cancers including breast (10, 11), esophageal (12, 13), lung (14, 15), prostate (16, 17), bladder (18, 19), skin (20, 21), and pancreas (22, 23).

Studies with specific inhibitors of COX-2 enzyme have shown significant effects in reducing the incidence and progression of tumors in both animal models and in treatment of cancer patients (6-8). Studies to evaluate effects of COX-2-specific inhibitors in the treatment of breast cancer have started recently; therefore, data are limited. In animal studies, COX-2 inhibitors have shown promising results. In rat models of chemical carcinogenesis, COX-2 inhibitors significantly reduced incidence and size of mammary tumors (31, 32). COX-2 inhibitors were also effective in retarding tumor progression and metastasis in mouse models of injected breast cancer cell lines and in xenograft models of human breast cancer cells in nude mice (24, 33, 34). Clinically, COX-2 inhibitors have been

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used in combination with other anticancer drugs or radiation therapy to treat solid tumors, mostly focusing on colon and colorectal cancers. Reports emerging from these studies strongly suggest that COX-2 inhibitors may emerge as a new generation of therapeutic drugs for cancer therapy. A recent report indicated that regular nonsteroidal anti-inflammatory drug use for 5 to 9 years was associated with a 21% reduction in the incidence of breast cancer and regular use for >10 years was associated with 28% reduction (35). This area of research is underexplored and more thorough preclinical trials are needed to further determine if COX-2 inhibition represents a useful approach to treatment of breast cancer.

Preclinical studies must precede clinical trials, and use of appropriate mouse models is key to the development of efficient therapeutic strategies. We have used in this study the oncogenic mice that carry the polyoma virus middle T antigen (MTag) driven by the mouse mammary tumor virus (MMTV) long terminal repeat promoter. These mice develop spontaneous tumors of the breast, which metastasize to the lungs and bone marrow. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MTag mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus MTag driven by the MMTV promoter (36). MTag specifically associates with and activates the tyrosine kinase activity of several c-*src* family members, eliciting tumors when a threshold level of gene product has been attained. In these mice, the MMTV promoter is transcriptionally active throughout all stages of mammary gland development, which results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Focal atypical lesions can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from ~60 days onward. Tumor progression is quite rapid, reaching 10% of body weight by ~20 to 24 weeks. All of the female mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast (37, 38). These mice exhibit metastasis in the lungs (60%) and micrometastasis in the bone marrow by 4 months of age (39). Therefore, the MTag mouse model is an appropriate model for human metastatic breast cancer in which to evaluate therapeutic strategies and to understand the mechanisms associated with therapy-induced growth inhibition. This is the first study to evaluate the efficacy and growth inhibitory mechanisms of celecoxib in an *in vivo* model of spontaneous metastatic breast cancer.

Results

Celecoxib Treatment Caused Significant Reduction in Primary Mammary Tumor Burden

Ten-week-old tumor-bearing female MTag mice were gavaged daily for 4 weeks with celecoxib at 5, 10, or 20 mg/kg body weight. In mice, at 10 and 20 mg/kg dose, the concentration of celecoxib in the plasma ranges from 6.5 to 13 μmol/L at 2 hours and from 4.2 to 8 μmol/L at 4 hours post-celecoxib treatment (40). This dose is attainable clinically and sufficient to inhibit PGE_2 (41). At 10 weeks, mice have small palpable tumors (1-2 tumors, ~0.1-0.5 mg tumor weight). One hundred percent of the MTag mice have hyperplastic mammary glands by 6 to 8 weeks (starting at time of puberty). Because the MTag is a strong oncogene, driven by the MMTV promoter, 100% of the MTag mice develop multifocal tumors with palpable tumors in at least 1 to 2 glands (of 10 mammary glands in mice) by 10 weeks. Every gland is hyperplastic by this time and every gland has palpable tumors by 14 weeks. Complete blood count analysis including hemoglobin levels was done to determine cytopenia and/or anemia post-celecoxib treatment. Regardless of the celecoxib dose, there was no detectable change in their complete blood count or hemoglobin levels (data not shown) as compared with untreated MTag mice. Flow cytometric analysis of T cells, B cells, and natural killer cells revealed no change in treated versus control MTag mice, nor were there any signs of weight loss in treated mice (data not shown). This suggested that celecoxib was well tolerated in these mice with no detectable signs of toxicity. Mice were sacrificed at 14 weeks of age, tumors were removed, and serum was collected. Tumor burden in MTag mice treated with 10 and 20 mg/kg dose was significantly reduced ($P < 0.003$ for 10 mg/kg and $P < 0.01$ for 20 mg/kg; Fig. 1). Note that in this study we started the treatment at 10 weeks when the mice had established tumors. The purpose of this study was to focus on the short-term effect of celecoxib on breast cancer cells *in vivo* at early times during tumor development and evaluate the mechanism of action of the drug on primary breast cancer cells. All mice were terminated at 14 weeks of age. The cumulative palpable tumors from 10 mammary glands at 14 weeks of age for individual mouse are presented in Fig. 1. Because metastasis in the MTag mice only develop between 19 and 24 weeks of age, we evaluated 6 MTag mice that received 20 mg/kg celecoxib and 10 vehicle-treated MTag mice between 20 and 24

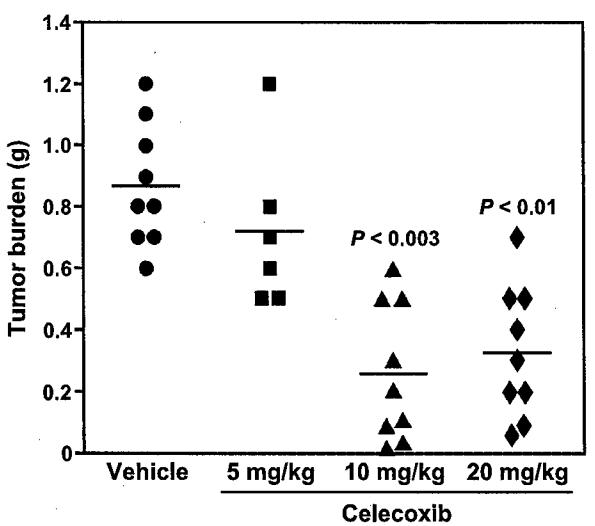

FIGURE 1. Reduced tumor burden in 14-week-old MTag mice post-celecoxib treatment. MTag mice were palpated weekly for presence of mammary tumors. Tumor weights plotted represent total tumor burden (including all mammary glands) per mice at 14 weeks of age ($n = 9$ mice for vehicle and 10 and 20 mg/kg celecoxib and $n = 6$ mice for 5 mg/kg celecoxib).

weeks of age. Gross microscopic examination of lungs revealed that the celecoxib-treated mice did not develop metastasis (0 of 6), whereas 6 of the 10 control mice developed lung metastasis (data not shown). These results are preliminary and we need to enroll more mice to the study to achieve statistical significance.

Celecoxib Induces Apoptosis in Breast Cancer Cells In vivo

We have reported recently that celecoxib induces growth inhibition of human and mouse breast cancer cells *in vitro* by simultaneously activating tumor cell apoptosis and inhibiting proliferation (42). Apoptosis of primary MTag tumor cells was determined by Annexin V/propidium iodide staining and flow cytometry. Data revealed significant increase in apoptotic cell population at 10 and 20 mg/kg celecoxib dose as compared with control MTag mice (39% in control mice versus 65% in 10 mg/kg dose, $P < 0.05$; 59% in 20 mg/kg dose, $P < 0.05$). The lowest dose (5 mg/kg) did not have a significant effect (Fig. 2A). Tumor cells from untreated MTag mice gave similar percentage of apoptotic cells (~35-40%) as vehicle-treated mice (data not shown). The high baseline apoptosis level in vehicle-treated and untreated mice is likely due to the method of isolating single cells. However, the 1.5- to 1.7-fold increase following celecoxib treatment was reproducibly observed.

We also evaluated celecoxib-induced apoptosis *in situ* by detection of DNA fragmentation using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (43). We observed an increase in TUNEL-positive cells in celecoxib-treated tumor sections *in situ* as compared with control tumor sections, confirming the flow cytometry data (Fig. 2B). Representative immunohistochemical images of vehicle-treated and celecoxib-treated MTag tumor sections are shown at 100× magnification, demonstrating considerable TUNEL positivity in celecoxib-treated versus control MTag tumor sections.

Increased Bax and Decreased Bcl-2 in Tumor Lysate Derived from Celecoxib-Treated MTag Mice

The downstream signaling pathways involved in COX-2-induced apoptosis are not well understood, but at least three pathways have been suggested: Bcl-2-mediated pathway, nitric oxide pathway, and production of ceramide (44). Because it has been shown previously in cell lines that celecoxib-induced apoptosis is associated with decreased Bcl-2 (an antiapoptotic protein) and increased Bax (a proapoptotic protein), we evaluated the levels of Bcl-2 and Bax by Western blot analysis of whole MTag tumor lysate post-celecoxib treatment. Treatment with celecoxib at 10 and 20 mg/kg induced increased expression of Bax (inducer of apoptosis) in all five mice tested as compared with vehicle-treated tumors (Fig. 3A). The increase was most pronounced at the 10 mg/kg dose of celecoxib. Simultaneously, there was decrease in Bcl-2 (inhibitor of apoptosis) protein expression in the 10 and 20 mg/kg dose of celecoxib (Fig. 3B). Untreated MTag tumor lysate was used as positive control in the first lane. These tumor lysates were prepared from 21-week-old MTag tumors, whereas the treated mice were at 14 weeks of age. This could explain the difference in protein expression observed

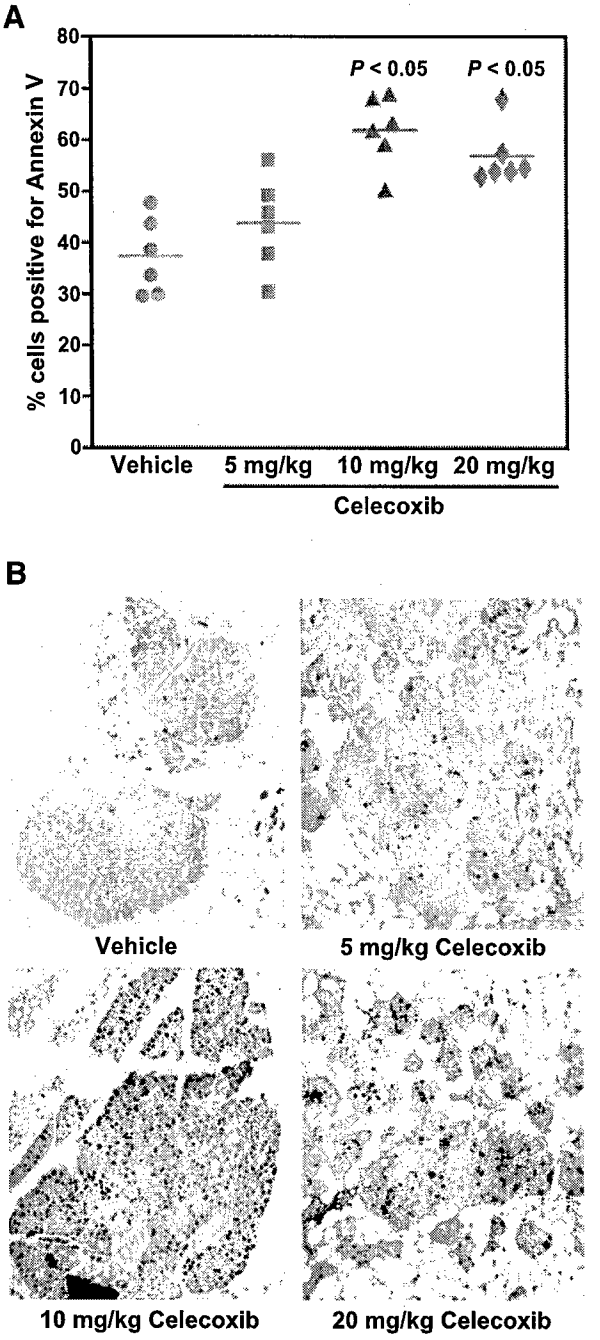

FIGURE 2. A. Increase in Annexin V–positive cells in celecoxib-treated MTag tumors *in vivo*. Tumor cells derived from vehicle-treated or celecoxib (5, 10, or 20 mg/kg body weight)–treated MTag mice were stained with Annexin V conjugated with FITC and propidium iodide, and percentage apoptotic cells (cells positive for Annexin V) were analyzed by flow cytometry. $n = 6$ mice per treatment group. *P*, significant difference between celecoxib-treated groups and vehicle control. **B.** Increase in TUNEL-positive cells in celecoxib-treated MTag tumors *in situ*. Light microscopic image of TUNEL-positive cells visualizing apoptosis *in situ* from mammary gland tumor sections isolated from vehicle and 5, 10, and 20 mg/kg celecoxib-treated MTag mice. *Brown*, apoptotic cells. All images are representative of five standardized fields from six separate mice. Magnification, ×100.

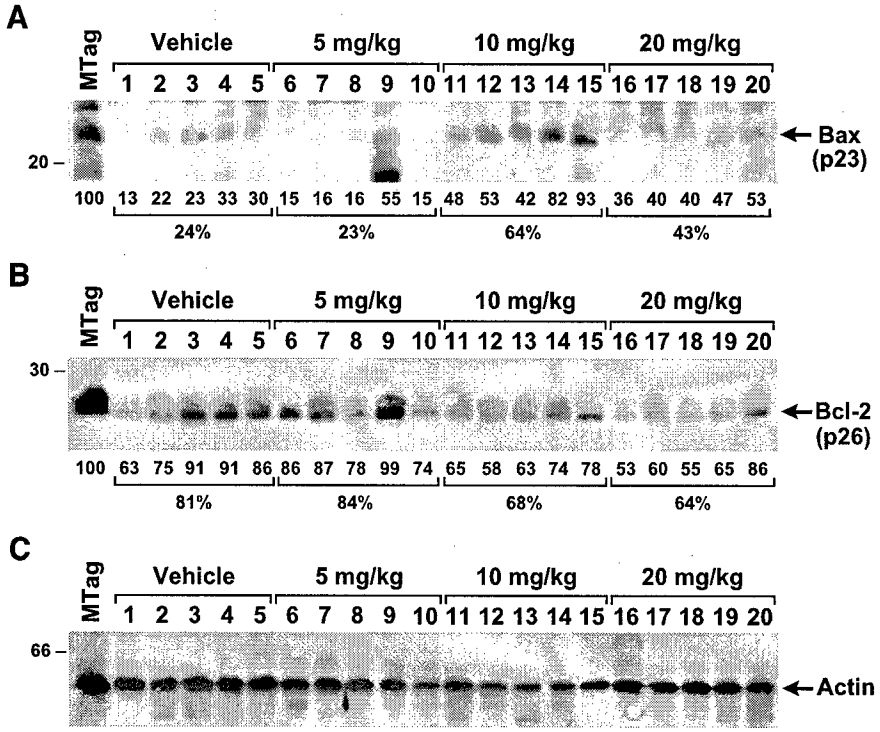

FIGURE 3. Increase in the proapoptotic protein Bax and decrease in the antiapoptotic protein Bcl-2 post-celecoxib treatment. Western blot analysis of Bax and Bcl-2 protein levels in mammary gland tumor lysates from vehicle and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice; 100 μg of protein were loaded per lane. $n = 5$ individual mice. *Numbers below each lane,* percentage of protein expression compared with MTag lysate, which was set to equivalent of 100% as determined by densitometric analysis. Average percentage expression for each treatment group ($n = 5$ mice). *P,* significant difference between treatment groups and vehicle control. β-Actin is used as the protein loading control for all tumor lysates.

between vehicle-treated and untreated MTag tumors. Tumor lysate from 14-week-old MTag mice have similar Bcl-2 and Bax levels as vehicle-treated tumors (data not shown). Densitometric analysis of the Western blots indicates significant increase in Bax protein levels between vehicle-treated and 10 mg/kg ($P < 0.05$) and 20 mg/kg ($P < 0.06$) celecoxib-treated tumor lysates. Similarly, significant decrease in Bcl-2 was observed between vehicle-treated and 5 mg/kg celecoxib–treated mice versus 10 mg/kg ($P < 0.05$) and 20 mg/kg ($P < 0.05$) celecoxib-treated groups. All comparisons are between 14-week-old vehicle-treated tumors and age-matched celecoxib-treated tumors. Thus, data suggest that celecoxib-induced apoptosis in MTag tumor cells *in vivo* is associated with an elevated expression of Bax and reduced expression of Bcl-2 proteins. These results give further credence to the flow cytometry and TUNEL data, confirming that celecoxib induces apoptosis *in vivo* in a highly aggressive and metastatic breast cancer model.

Reduced Phosphorylation of Akt in Tumor Lysate Derived from Celecoxib-Treated MTag Mice

Protein kinase B/Akt is a serine/threonine protein kinase that is involved in promoting cell survival signals through the phosphatidylinositol 3-kinase (PI3K) pathway leading to inactivation of a series of proapoptotic proteins. These kinase activities are frequently deregulated in human disease including cancer (45). Akt represents a key signaling component in cell survival by activating downstream proapoptotic proteins and caspases (46-48). Celecoxib has been shown recently to induce apoptosis of cancer cells by blocking Akt activation in cultured prostate cancer cells (49, 50). To explore whether inhibition of Akt activation may be linked to the observed *in vivo* apoptosis in MTag tumors, we determined the effect of *in vivo* celecoxib administration on phosphorylation of Akt (at Ser^{473} in the carboxyl terminus) in MTag tumors. Data show that celecoxib substantially suppresses phosphorylation of Akt in MTag tumors. Two of five mice in the 5 mg/kg dose showed reduced phosphorylation, whereas four of five in 10 mg/kg and five of five mice in 20 mg/kg dose showed reduced Akt activation (Fig. 4). Densitometric analysis clearly indicates significant down-regulation of Akt phosphorylation in celecoxib-treated tumors as compared with vehicle-treated tumors ($P < 0.05$ for 10 and 20 mg/kg celecoxib). All tumors showed approximately equivalent levels of the Akt protein as shown in Fig. 4 (*bottom*). This result clearly suggested the involvement of the Akt pathway in induction of apoptosis *in vivo* in our mouse model of spontaneous breast cancer. Akt represents a key signaling component in cell survival by activating downstream proapoptotic proteins and caspases (46-48). Because we observed a decrease in Akt phosphorylation and increase in proapoptotic protein (Bax), we determined if caspases were activated post-celecoxib treatment. MTag tumor cells treated with celecoxib *in vitro* (20, 40, and 60 μmol/L) were analyzed for activation of effector caspase-3 and caspase-7. Most apoptotic signals induce intracellular cleavage of caspase-3 and caspase-7 and convert them into active forms. Caspase activity is presented as fluorescence emission, which is directly proportional to caspase-3/7 activities. Increase in fluorescence emission was observed with increasing dose of celecoxib, which correlates with increase in active forms of caspase-3 and caspase-7. Table 1 illustrates the fluorescence emission for untreated versus

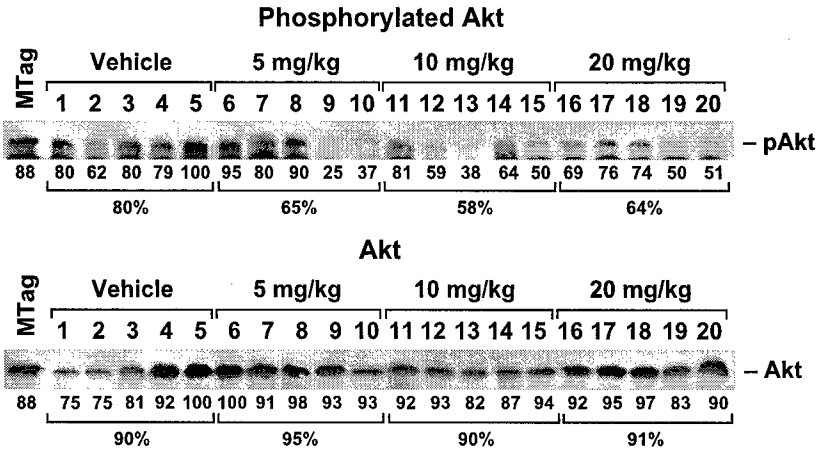

FIGURE 4. Decreased phosphorylation of protein kinase B/Akt post-celecoxib treatment. Western blot analysis of phospho-Akt (*pAkt*) and Akt protein levels in mammary gland tumor lysates from vehicle and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice; 100 μg of protein were loaded per lane. $n = 5$ individual mice. *Numbers below each lane,* percentage of protein expression compared with mouse expressing the most protein, which was set equivalent to 100% as determined by densitometric analysis. Average percentage expression for each treatment group ($n = 5$ mice). *P,* significant difference between treatment groups and vehicle control.

celecoxib-treated MTag tumor cells. Significant increase in fluorescence emission was observed in 40 and 60 μmol/L celecoxib-treated cells compared with untreated or vehicle-treated cells ($P < 0.05$ and 0.01, respectively).

Celecoxib Inhibits Tumor Cell Proliferation

Antiproliferative effect of *in vivo* celecoxib treatment was determined by *in situ* immunohistochemical analysis of MTag tumor sections stained with proliferating cell nuclear antigen (PCNA). A representative light microscope image suggests inhibition of proliferation in MTag tumors *in vivo* with 10 and 20 mg/kg celecoxib treatment (Fig. 5). PCNA protein levels peak during the S phase of the cell cycle and is almost undetectable in other phases of the cycle. Vehicle and 5 mg/kg celecoxib treatments show almost every cell expressing PCNA, indicative of highly proliferative cells (Fig. 5A and B). With 10 and 20 mg/kg treatment, fewer cells expressed PCNA staining, suggestive of fewer cells undergoing proliferation (Fig. 5C and D). A lymph node within an untreated mammary tumor section shows only a few proliferating cells, confirming the specificity of the stain (Fig. 5E). Staining specificity is further confirmed with second antibody control (Fig. 5F).

Celecoxib Treatment Significantly Reduced Serum Levels of PGE_2 In vivo

Next, we analyzed sera and tumor lysate from celecoxib-treated and vehicle-treated MTag mice for PGE_2 levels to assess COX-2 activity *in vivo*. COX-2 converts arachidonic acid to bioactive prostaglandins. It has been shown that COX-2-derived PGE_2 is the major prostaglandin produced by breast cancer cells and may be required for the angiogenic switch leading to initiation and progression of mammary cancer in a MMTV-COX-2 transgenic mouse model (51). Production of secreted PGE_2 is an appropriate measure of COX-2 activity in the MTag mouse model. PGE_2 is unstable *in vivo* and

Table 1. Caspase-3 and Caspase-7 Activity of MTag Cells Treated with Celecoxib

Treatment	Caspase-3/7 Activity (Fluorescence Emission)
Vehicle	$1 \times 10^6 \pm 2.0 \times 10^5$
Celecoxib (20 μmol/L)	$2 \times 10^6 \pm 1.6 \times 10^5$
Celecoxib (40 μmol/L)	$4 \times 10^6 \pm 2.2 \times 10^5$*
Celecoxib (60 μmol/L)	$5 \times 10^6 \pm 2.0 \times 10^5$*

NOTE: Spectrofluorometric analysis of lysates prepared from 48-hour vehicle-treated and celecoxib (20, 40, and 60 μmol/L)–treated MTag cells. Activity of caspase-3 and caspase-7 was monitored by enzymatic cleavage using a fluorescence microplate reader with excitation at 485 ± 10 nm and emission detection at 530 ± 12.5 nm. Mean ± SD of three experiments.
*$P < 0.01$, significant difference between vehicle control and celecoxib treatment.

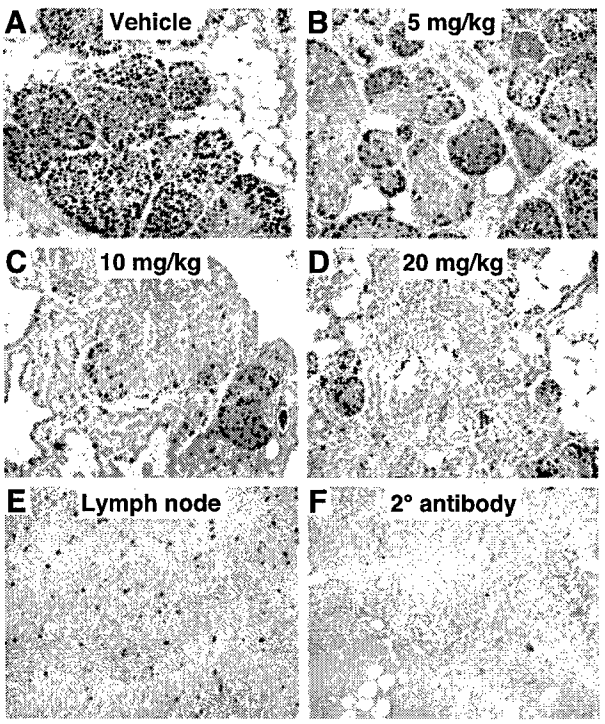

FIGURE 5. Celecoxib-induced inhibition of tumor cell proliferation *in vivo* in a dose-dependent manner. Light microscopy images of PCNA staining of mammary tumor sections from vehicle-treated (**A**) and celecoxib (5, 10, and 20 mg/kg)–treated (**B-D**) MTag mice. All images are representative of five standardized fields from six separate experiments. Inhibition of proliferation is most evident at 10 and 20 mg/kg dose of celecoxib. Lymph node section (**E**) and second antibody staining (**F**) are shown as controls. Magnification, ×200.

measurement of the metabolites is necessary to provide a reliable estimate of actual PGE_2 production. Thus, we measured PGE_2 metabolite (PGEM; i.e., 13,14-dihydro-15-keto prostaglandin A_2) using a commercially available ELISA. A significant reduction in serum PGEM is observed in 10 and 20 mg/kg celecoxib-treated MTag mice as compared with pretreatment and vehicle-treated mice (2,000 pg/mL in vehicle-treated mice versus <1,000 pg/mL in 10 mg/kg celecoxib-treated mice, $P < 0.01$; Fig. 6A). Similar reduction in PGEM was observed in tumor lysates (data not shown). Note that the serum PGEM levels never reached the values observed in nontumor C57BL/6 mice of 300 pg/mL (Fig. 6A). This suggests that, although celecoxib was partially effective in reducing PGEM levels, treatment was not sufficient to completely reverse the up-regulation of PGE_2 levels in MTag mice because these mice were not completely tumor free.

To test if celecoxib had a direct effect on COX-2 protein expression in the tumor, we evaluated COX-2 protein expression in tumor lysates from vehicle-treated and celecoxib-treated MTag tumors. MTag tumors from untreated and vehicle-treated mice expressed higher levels of COX-2 (Fig. 6B) as compared with celecoxib-treated (10 and 20 mg/kg) tumors, indicating that celecoxib has a direct effect on COX-2 protein expression *in vivo*. Densitometric analysis showed some difference between vehicle-treated and 10 or 20 mg/kg celecoxib-treated tumors ($P < 0.07$). However, the direct effect of celecoxib on COX-2 protein expression was less significant ($P < 0.07$) than its effect on COX-2 activity as measured by PGE_2 levels.

Celecoxib Treatment Reduced Vascular Endothelial Growth Factor Levels In vivo

It has been shown recently that COX-2-induced PGE_2 stimulated the expression of angiogenic regulatory genes including vascular endothelial growth factor (VEGF) in mammary tumor cells isolated from COX-2 transgenic mice and that treatment with indomethacin (nonspecific COX inhibitor) suppressed the expression of these genes *in vitro* (51). We therefore evaluated levels of *in vivo* VEGF protein levels in the tumor microenvironment of MTag tumors post-celecoxib treatment. Treatment with celecoxib (10 or 20 mg/kg) reduced VEGF levels in the tumor lysate in four of six treated mice as compared with vehicle-treated MTag tumors ($P < 0.05$; Fig. 7). No reduction was observed in mice treated with 5 mg/kg celecoxib. Untreated MTag tumor lysate had similar levels as vehicle-treated MTag mice (data not shown). Similar reduction in circulating VEGF levels was also

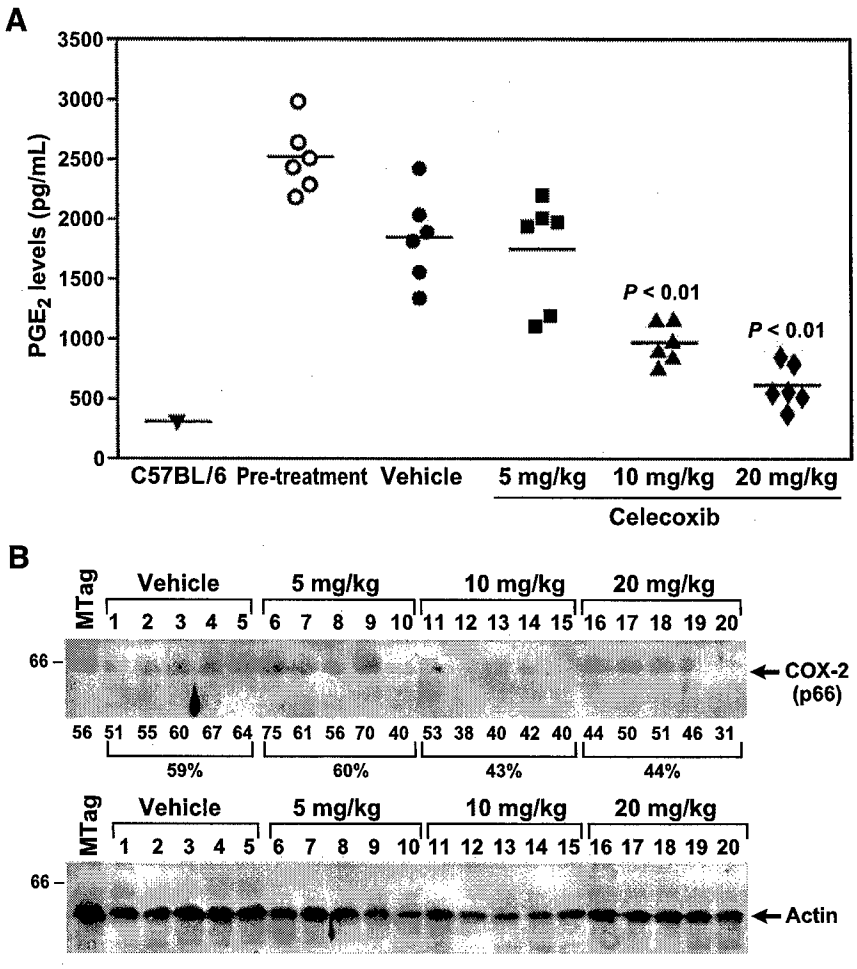

FIGURE 6. A. Dose-dependent inhibition of PGE_2 synthesis in serum of celecoxib-treated MTag mice. PGEM levels in serum were determined using specific ELISA (pg/mL serum). Serum from mice was collected either before treatment commenced or after 4 weeks of celecoxib treatment. *P*, significant difference between celecoxib-treated and untreated (pretreatment) or vehicle-treated mice. PGE_2 levels are also compared with serum from age-matched non-tumor-bearing wild-type C57BL/6 mice. Significant inhibition is evident at 10 and 20 mg/kg dose. **B.** Decrease in COX-2 protein expression is not significant in MTag tumors post-celecoxib treatment ($n = 5$ mice). Western blot analysis of tumor lysates from untreated, vehicle-treated, and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice; 100 μg of protein were loaded per lane. All tumor lysates expressed COX-2. Untreated and vehicle-treated tumors expressed higher levels of COX-2 than tumor lysates from celecoxib (10 and 20 mg/kg)–treated mice. *Numbers below each lane*, percentage of protein expression compared with mouse expressing the most protein, which was set equivalent to 100% as determined by densitometric analysis. Average percentage expression for each treatment group ($n = 5$ mice). β-Actin is used as the protein loading control for all tumor lysates.

observed. Serum levels of VEGF in untreated or vehicle-treated 14-week-old MTag mice were found to be between 150 and 400 pg/mL, whereas in the mice treated with celecoxib (10 or 20 mg/kg) the levels ranged from 20 to 90 pg/mL. In some of the treated mice, the VEGF levels were too low to be detected by ELISA. Preliminary histologic evaluation also suggests the presence of fewer blood vessels in the celecoxib-treated tumor sections versus control tumor using the Masson's trichrome staining. A representative picture of vehicle, 5, 10, and 20 mg/kg celecoxib-treated MTag tumor is shown in Fig. 7B.

Discussion

We show for the first time that *in vivo* treatment with celecoxib causes significant reduction in mammary gland tumor burden in a mouse model of spontaneous breast cancer. Recently, we have evaluated 6 MTag mice that received 20 mg/kg celecoxib and 10 control MTag mice between 20 and 24 weeks of age. None of the celecoxib-treated mice developed lung metastasis, whereas 5 of the 10 control mice developed lung metastasis (data not shown).

Tumor reduction was associated with induction of tumor cell apoptosis *in vivo*. Investigation into the potential molecular pathway revealed that treatment with celecoxib caused reduction in activation of antiapoptotic/prosurvival kinase (Akt). Increased apoptosis was associated with increased expression of the proapoptotic protein Bax and decreased expression of the antiapoptotic protein Bcl-2. Concurrently, we observed decreased tumor cell proliferation and decreased synthesis of VEGF in mammary gland tumors treated with celecoxib *in vivo*, most probably associated with decreased PGE_2 synthesis.

The importance of this study lies in the use of a mouse model system that resembles human disease in many aspects of tumor progression. The MTag tumors start as hyperplasia, like early proliferative lesions seen in the human breast; show indication of histologic progression to malignant mammary adenocarcinomas and metastasis; are heterogenous with respect their malignant potential; and trigger signaling pathways inactive in normal breast epithelium (38). One of the pathways that is activated in these mice is the arachidonic acid/COX-2 pathway (52), similar to that described in many human breast cancers. Furthermore, we have shown recently that COX-2 protein and its downstream product PGE_2 were highly elevated in human breast tumors and lymph node metastasis compared with normal tissue, with the highest expression being observed in lymph node metastasis (53). There was a direct correlation between increased COX-2 and PGE_2 expression with impaired immune cell function in newly diagnosed stage I and II breast cancer patients (53). Our observations are similar to the reports that have shown significant elevation of COX-2 protein levels in 43% of human invasive breast cancers and 63% of ductal carcinomas *in situ* (11, 54). Thus, the MTag model offers the potential to evaluate chemoprevention with a highly specific COX-2 inhibitor, celecoxib.

Celecoxib has been shown to target multiple pathways of tumorigenesis including proliferation, apoptosis, angiogenesis, invasion, and tumor-induced immune suppression in various

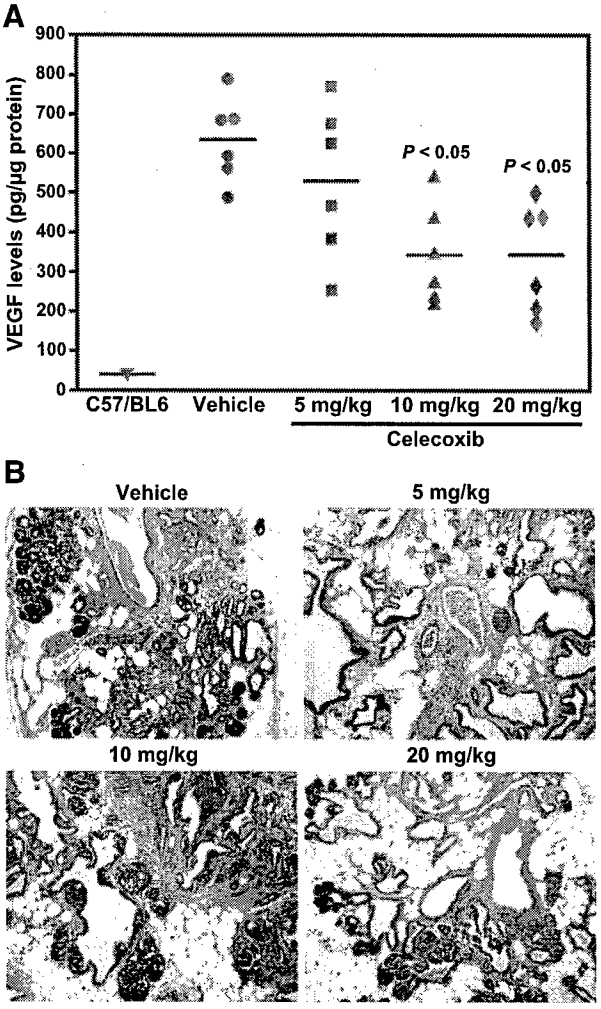

FIGURE 7. A. Decreased VEGF levels in MTag tumors from celecoxib-treated mice. VEGF levels were determined in the tumor lysates using specific ELISA (pg/μg protein lysate). Tumor lysate was prepared from untreated, vehicle-treated, and celecoxib (5, 10, and 20 mg/kg)–treated MTag mice. *P*, significant difference between celecoxib-treated and vehicle-treated mice. Values are also compared with mammary gland lysate from age-matched non-tumor-bearing wild-type C57BL/6 mice. Significant inhibition is evident at 10 and 20 mg/kg dose. **B.** Fewer blood vessels in 10 and 20 mg/kg celecoxib-treated tumor sections compared with vehicle-treated tumor. Blood vessels in MTag tumor sections were histologically evaluated by Masson's trichrome. This method stains fibrous tissue and stroma green. Blood vessels containing RBC stain bright red. Fewer blood vessels in the tumor section of the celecoxib-treated tumors (10 and 20 mg/kg) relative to those obtained from vehicle-treated animals. Magnification, ×100.

breast tumor cell lines. The current report by Chang et al. (51) supports the concept that COX-2 may provide an early target for breast cancer prevention. We show that early intervention with celecoxib causes reduced primary tumor burden in the MTag model (Fig. 1). We further show that reduced PGE_2 synthesis (Fig. 6A) and reduced PI3K/Akt kinase activation (Fig. 4) post-celecoxib treatment may be the mechanism(s) underlying enhanced tumor cell apoptosis (Fig. 2) and reduced tumor cell proliferation (Fig. 5) *in vivo*. Our data are in line with the recent *in vitro* study in prostate cancer cell lines, where

it was shown that celecoxib induces apoptosis by blocking or suppressing Akt activation (50). The PI3K/Akt pathway is typically activated in response to oncogenes that bind to receptor kinases at the plasma membrane and lead to the activation of PI3K (55, 56). Activated Akt targets multiple factors involved in cell proliferation, migration, and survival/apoptosis. Mechanistically, activated Akt is known to trigger several cyclins including cyclin D1 that affects all stages of the cell cycle and induces downstream proliferation (56). Preliminary data suggest decreased levels of cyclin D1 in tumor lysates of mice treated with celecoxib, with significant arrest of the mammary tumor cells at the G_2-M checkpoint phase of cell cycle (data not shown). Thus far, our results implicate the PI3K/Akt pathway to be critical in the celecoxib-induced apoptosis and inhibition of tumor cell proliferation. However, other pathways such as the Raf/mitogen-activated protein kinase-extracellular signal-regulated kinase/mitogen-activated protein kinase pathway may also be affected by celecoxib, and future studies will be designed to evaluate these pathways *in vivo* in the MTag mouse model. One potential mechanism that has been associated with PGE_2-related inhibition of apoptosis is that PGE_2 reduces the basal apoptotic rate by increasing the level of antiapoptotic proteins such as Bcl-2 (54, 57). Our *in vivo* data support this concept, because inhibiting PGE_2 production by targeting COX-2 activity in the MTag tumors led to decrease in Bcl-2 protein levels and concurrent increase in the proapoptotic protein Bax (Fig. 3A and B) as well as activate effector caspase-3 and caspase-7 (Table 1).

Finally, angiogenesis plays a crucial role in tumor development and progression. COX-2-dependent PGE_2 is a potent inducer of angiogenesis *in vivo* and induces expression of angiogenic regulatory proteins such as VEGF (51, 58, 59). It has been shown recently that overexpression of COX-2 in the mammary gland by MMTV promoter induces mammary carcinogenesis and that the major prostaglandin that is produced in these tumors is PGE_2 (51, 54). These authors further defined the role of COX-2-dependent PGE_2 production in transforming local tumors to invasive cancer by triggering a so-called angiogenic switch by increasing expression of proangiogenic mediators such as VEGF and its receptors. Thus, we examined whether celecoxib treatment *in vivo* was effective in reducing the exaggerated VEGF levels observed in MTag tumors and in the serum. Significant decrease in levels of VEGF in the mammary gland tumors accompanied by fewer blood vessels in the celecoxib-treated tumor sections versus control was observed (Fig. 7), once again suggesting a role of COX-2 and PGE_2 in mediating angiogenesis in the polyoma virus MTag-induced breast tumors. Although additional mechanisms are involved in mediating the angiogenic effects of COX-2, our data suggest that COX-2 influences angiogenesis at least in part by enhancing VEGF secretion by tumor endothelial cells. Additional studies are needed to fully elucidate the complex events involved in COX-2-mediated angiogenesis in our model. Our data clearly show extensive down-regulation of PGE_2 in serum (Fig. 6A) post-celecoxib treatment *in vivo*. PGE_2 binds to cell surface receptors that belong to the family of seven-transmembrane domain G protein-coupled receptors, designated EP1, EP2, EP3, and EP4 (54, 60). Future studies will determine the pattern of prostanoid receptor distribution in the MMTV-MTag mice and whether COX-2 inhibitors can modulate prostanoid receptor expression and its activation state. Although we suggest that PGE_2 down-regulation may be in part responsible for the reduced VEGF levels, we fully recognize that much work is required to define a direct relationship between PGE_2 and VEGF and that other pathways and angiogenic markers may be involved. We also acknowledge that the effect of celecoxib in the MTag mice may be COX independent; indeed, we do not see a dramatic down-regulation of COX-2 expression in celecoxib-treated compared with vehicle-treated mice (Fig. 6B). However, we must point out that we have published that the MTag tumors overexpress COX-2 and the expression increases as tumors progress and that the PGE_2 levels are significantly decreased with celecoxib treatment.

In summary, celecoxib treatment may exert its antiproliferative, antiangiogenic, and proapoptotic effects by regulating the PGE_2-prostanoid receptor–associated pathways and by decreasing PI3K/Akt phosphorylation. This leads to significant reduction in primary breast tumor burden. Furthermore, this effect may or may not be dependent on down-regulation of COX-2 protein expression in the tumor. Thus, we believe that COX-2 inhibitors not only represents a future therapeutic option for the treatment of human breast cancer in combination with standard therapies but also may be considered as a potent chemopreventive agent for individuals with high risk of developing breast cancer and for individuals with high risk of disease relapse.

Materials and Methods

Generation of MTag Mouse Model

MTag oncogenic mice was originally a kind gift from Dr. W.J. Muller (McGill University, Toronto, Ontario, Canada; ref. 36). MTag male mice were mated to C57BL/6 mice to maintain the MTag mice as heterozygous. Approximately 50% of the pups carry the oncogene, and in these pups, ~50% are females that develop mammary gland adenocarcinomas and are used for the experiments. PCR was used to routinely identify the MTag oncogene. PCR was carried out as described previously (39). Primer pairs for MTag transgene are 5′-AGTCACTGCTACTGCACCCAG-3′ (282-302 bp) and 5′-CTCTCCTCAGTTCCTCGCTCC-3′ (817-837 bp). The amplification program for MTag consisted of 1 cycle of 5 minutes at 95°C and 40 cycles of 30 seconds at 95°C, 1 minute at 61°C, and 30 seconds at 72°C followed by 1 cycle of 10 minutes at 72°C. The PCR product was analyzed by size fractionation through a 1% agarose gel. Amplification of MTag gene results in a 480-bp fragment. All mice are congenic on the C57/BL6 background at $n \geq 10$. All mice were bred and maintained in specific pathogen-free conditions in the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility. All experimental procedures were conducted according to Institutional Animal Care and Use Committee guidelines.

Celecoxib Treatment

Celecoxib was purchased from Pharmacia Pharmaceuticals (Skokie, IL) as 100-mg capsules. Drug was prepared for p.o. administration according to the manufacturer's recommendation.

Briefly, the drug was dissolved in DMSO, rotated at low speed in a 37°C hot room for 12 hours, and centrifuged at 1,800 rpm for 10 minutes, and the supernatant was collected and stored at 4°C as stock solution of 20 mg/mL. Ten-week-old female MTag mice were gavaged p.o. with 20-gauge barrel tip feeding needles (Fine Science Tools, Foster City, CA) at 5, 10, or 20 mg/kg body weight daily (5 days on with 2 days off) for 4 weeks. Control mice were gavaged with DMSO. Six mice per treatment group were used. Following 4 weeks of treatment, mice were sacrificed and mammary tumors dissected and divided into three parts: (*a*) to generate single cell suspension for flow cytometry, (*b*) to prepare tumor lysate for Western blot analysis and ELISA, and (*c*) to fix in formalin and embedded in paraffin blocks for immunohistochemical analysis. Serum was collected for ELISA. A dose range of 5 to 20 mg/kg body weight was used in our spontaneous mouse model based on previous reports in the literature (24, 34). These doses correspond to physiologic dose of celecoxib and are clinically relevant because the doses of COX-2 inhibitors recommended to patients are in the range of 5 to 20 mg/kg body weight (29).

Tumor Burden

From 10 weeks of age until sacrifice, control and celecoxib-treated mice were palpated weekly for presence of mammary tumors. Palpable tumors were measured by calipers and tumor weight was calculated according to the following formula: $g = L$ (cm) $\times$ W (cm^2) / 2 (39).

Analysis of Apoptosis by Flow Cytometry

Part of the tumor tissue was dissociated to generate single cell suspension by incubating in 5 mmol/L EDTA solution for 1 hour at 37°C. Apoptosis was determined by staining single cells (1×10^6) with Annexin V and propidium iodide using the BD PharMingen (San Diego, CA) apoptosis kit following the manufacturer's protocol. Cell staining was determined by flow cytometry using the CellQuest program. Percentage apoptotic cells were determined by CellQuest statistical analysis program as the cumulative percentage cells that were stained positive for both propidium iodide and Annexin V (upper right quadrant) and cells that were stained for Annexin V only (lower right quadrant).

Analysis of Apoptosis, Proliferation, and Blood Vessels by Immunohistochemistry

Part of the tumor was formalin fixed [10% neutral-buffered formalin (pH 6.8-7.2), Fisher Scientific, Pittsburgh, PA] and paraffin embedded and 5-μm sections were prepared by the Mayo Clinic Scottsdale Histology Core Facility. Immunohistochemistry was done using the ApopTag Peroxidase *In situ* Apoptosis Detection kit (Serologicals Corp., Norcross, GA). 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. TUNEL-positive cells were examined under light microscopy and representative images taken at 200×. For PCNA staining, paraffin-embedded and 5-μm sections were subjected to antigen retrieval using the DAKO Target Retrieval (Carpinteria, CA) at 95°C for 40 minutes. Primary antibody (PCNA antibody, BD Biosciences, San Jose, CA) was used at 5 μg/mL at 4°C overnight and DAKO anti-mouse secondary conjugated to horseradish peroxidase was used at 1:200 for 2 hours at room temperature. 3,3′-Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. Histologic evaluation of vascularity was determined by Masson's trichrome staining (61). This method stains fibrous tissue and stroma green. Blood vessels containing RBC stain bright red.

Assay for Caspase-3 and Caspase-7

Primary MTag tumor cells derived from 17-week-old MTag mice were treated with increasing concentrations (20-60 μmol/L) of celecoxib or DMSO (vehicle) in medium supplemented with 5% FCS for 48 hours. To evaluate if celecoxib treatment can induce activation of caspase-3 and caspase-7, we detected levels of active forms of caspase-3 and caspase-7 in freshly prepared cell lysates from treated and untreated MTag tumor cells using the EnzChek Caspase-3/7 Assay Kit (Molecular Probes, Eugene, OR) following the manufacturer's protocol. In principle, active caspase-3 or caspase-7 will cleave a fluorogenic substrate releasing the fluorochrome, and the fluorescence was detected and quantified by spectrofluorometry using UV excitation of 380 nm and detected at an emission wavelength range of 430 to 460 nm. Fluorescence emission is an indication of caspase-3 and caspase-7 activity. Thus, apoptotic cell lysates containing active caspase-3 and caspase-7 yield considerable emission as compared with nonapoptotic lysates that do not contain the active forms of the enzymes.

ELISA for PGE_2 and VEGF

PGE_2 and VEGF enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI for PGE_2 and Oncogene Research Products, La Jolla, CA for VEGF) were used to assay the levels of PGE_2 and VEGF in tumor lysates and serum derived from treated and control mice. All tumor lysates were made in tissue lysis buffer containing 20 mmol/L HEPES, 0.15 mol/L NaCl, and 1% Triton X-100 supplemented with 80 μL/mL phosphatase inhibitor cocktail II (Sigma P-5726, St. Louis, MO) and 10 μL/mL complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Indianapolis, IN). The PGE_2 and VEGF assays were done according to the manufacturer's recommendation. Lysates were diluted appropriately to ensure that readings were within the limits of accurate detection. Results are expressed as picogram of PGE_2 or VEGF per milliliter of serum or per microgram protein of tumor lysate for individual mice.

Western Blot Analysis for COX-2, Phospho-Akt, Bax, and Bcl-2

Tumor lysates from treated and untreated mice prepared as stated previously were resolved by SDS-PAGE on 10% to 15% resolving gels. Tumor lysate (100 μg) was loaded per lane. Gels were blotted and probed for COX-2 (p70, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Akt and Akt protein (p60, 1:1,000, Cell Signaling, Beverly, MA), Bax-horseradish peroxidase conjugated (p23, 1:200, Santa Cruz Biotechnology), and Bcl-2 (p26, 1:1,000, Trevigen, Gaithersburg, MD). Mammary gland tumor lysates from 20- to 22-week-old MTag

mouse are used as positive control for COX-2. Jurkat T lymphoma cell lysate was used as positive control for the other proteins. Individual animal protein expression data are shown.

Statistical Analysis

All data are expressed as means ± SD. Statistically significant difference between experimental groups was assessed by one-way ANOVA with Dunnett adjustment.

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Mayo Clinic Cancer Center

MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas

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Protocol Resources

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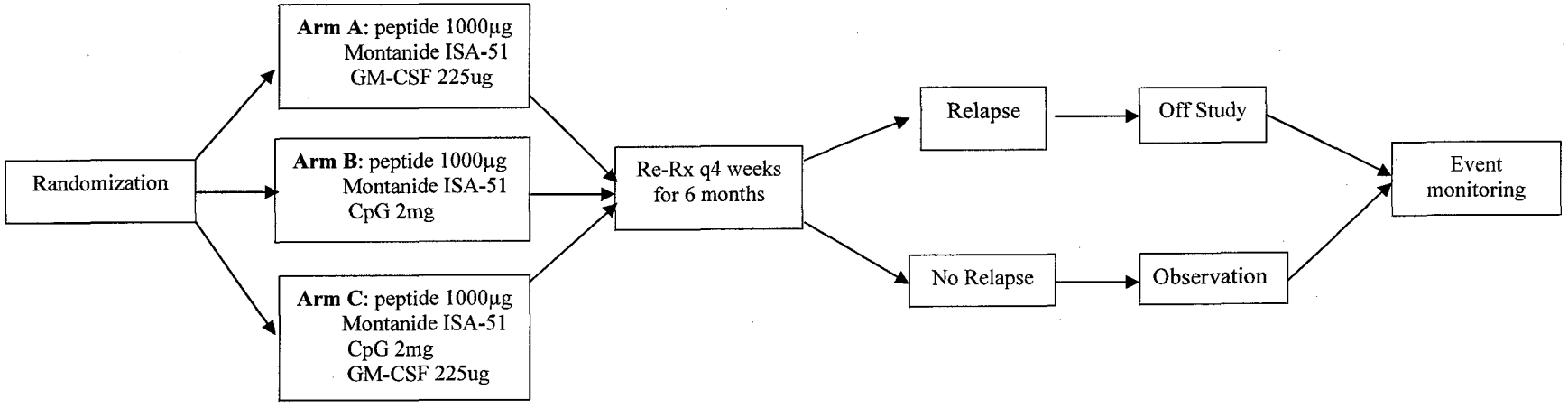

Randomization
Arm A: peptide 1000µg
Montanide ISA-51
GM-CSF 225ug
Arm B: peptide 1000µg
Montanide ISA-51
CpG 2mg
Arm C: peptide 1000µg
Montanide ISA-51
CpG 2mg
GM-CSF 225ug
Re-Rx q4 weeks
for 6 months
Relapse
Off Study
No Relapse
Observation
Event
monitoring

1.0 Background

1.1 Breast cancer is diagnosed in 200,000 individuals in the United States annually and contributes to approximately 40,000 deaths each year. For tumors confined to the breast, surgical removal provides a good prognosis. However, primary tumor that metastasizes to distant sites, such as lymph nodes, lungs, liver and brain, correlates with a poor prognosis. Patients with advanced stage breast cancer are at high risk of relapse. Complications from metastatic disease are the leading causes of cancer-related deaths. Novel adjuvant strategies, such as breast cancer specific vaccines, are being considered as a clinical intervention that may reduce the chance of recurrence.

In recent years there has been great interest in the development of these cancer vaccines, which are designed to immunize individuals to antigens present on tumors. Cancer vaccines are a non-toxic therapy, which have been shown in several melanoma trials to have the potential of controlling disease and prolonging survival because tumors can be surgically removed and there is often a long period of time before the tumor recurs at metastatic sites, cancer vaccines have been proposed as an optimal therapy that could prolong the time to recurrence. This optimal opportunity of immunization in the situation of minimal residual disease has rarely been tested, however, as most vaccines have been given to patients with large tumor burden after the failure of standard therapies in Phase I and Phase II trials. Recently, several groups have addressed the use of adjuvant immunotherapy following complete surgical resection [1]. Data from these studies are not yet complete.

1.2 The past two decades in tumor immunology have led to the discovery of specific tumor antigens that have been shown in preclinical studies to elicit tumor-specific immunity and establish long term memory without autoimmunity. For breast cancer, vaccines composed of epitopes derived of MUC1, HER-2/neu, MAGE3, CEA have been studied and shown to be immunogenic without causing autoimmunity [2-5].

1.3 It is now clear that tumor antigens are presented in the context of specific class I and Class II HLA molecules. Class I presentation, in the presence of appropriate co-stimulation, is thought to stimulate a cytolytic $CD8^+$ T cell response, while antigen presentation in the context of Class II molecules stimulates a $CD4^+$ helper T cell response [6].

1.4 One approach for the development of a cancer vaccine is the use of tumor associated synthetic antigens for immunologic priming. Because specific peptides are ubiquitous in tumors of the same histologic type, identical peptide vaccines may be employed in allogeneic hosts bearing the same tumor histology. Additionally, the use of single peptides for immunization limits the potential induction of undesired autoimmunity [7-9]. Recent developments in the use of soluble MHC Class I/peptide tetramers and elispot technology have enabled rapid characterization of epitope-specific CTL responses [10, 11]. In addition to being well-explored and understood, many of these antigens are shared tumor antigens. Vaccines that are composed of these antigens can be developed for use in a large number of patients. The primary limitations to peptide based vaccine strategies are haplotype restriction, potential for degradation, and uncertainty regarding which peptides, used alone or in combination, are the most immunogenic [12, 13]. This study is designed to test these uncertainties.

1.5 One attractive and broadly applicable target for immunotherapeutic strategies is the MUC1 tumor antigen. MUC1, a cell-associated mucin, is expressed on the cell surface of many epithelial malignancies as well as by hematological malignancies [14-17]. These include multiple myeloma (92%) and acute myelogenous leukemia (67%) [18]. Greater than 90% of breast carcinomas express MUC1; high levels are also found in adenocarcinomas originating from most tissues [14, 16]. MUC1 expression is greatly up-regulated on tumors (reviewed in

Gendler [19]). Expression on tumors is no longer apical, but it is found all around the cell surface and in the cytoplasm. In addition, glycosylation on tumor-synthesized MUC1 is aberrant, with greater exposure of the peptide core than is found in normal tissues. MUC1 has long been an interesting target molecule for immunotherapeutic strategies, given its high level and ubiquitous expression. Patients with tumors, especially with breast, pancreas and ovarian tumors, have exhibited immune responses to MUC1 with the presence of antibodies and T cells specific for MUC1 detected in about 10% of individuals. An HLA unrestricted T cell response among cancer patients has also been described [20-23]. There is increasing evidence from murine and human studies that MHC-restricted T cells can be induced in mice and humans after immunization with the MUC1 peptide or MUC1 antigenic epitopes [24-32]. Importantly, there have been reports of two HLA-A2 binding peptides derived from the MUC1 protein [33]. One of the peptides is from the tandem repeat sequence of MUC1 and the second peptide is from the signal sequence. MUC1-specific cytotoxic T cells (CTLs) have been induced in T cells from healthy donors following *in vitro* immunization using peptide-pulsed dendritic cells. MUC1-specific CTLs have also been induced *in vivo* after vaccination of breast and ovarian cancer patients with peptide-pulsed DCs [18].

1.6 A second candidate for peptide-based immunotherapy is HER-2/neu, the gene product of the erbB2/neu protooncogene. HER-2/neu is overexpressed in approximately 30% of breast cancer patients. HER-2/neu is also expressed by multiple types of tumors, including ovarian, lung, colon, pancreas and gastric tumors [34-36]. HER-2/neu has particular relevance, as it is expressed at high levels in early *in situ* lesions in breast carcinoma [37]. Thus, it is a target for early disease. Immunologic responses to HER-2/neu have been detected in a minority of patients with advanced stage breast and ovarian cancer, including antibodies, T helper and CD8 responses [38, 39]. Several HLA-class I binding peptides have been previously identified. A novel HLA-A2.1 binding peptide from the HER-2/neu extracellular domain [HER-2(9_{435})] was recently identified [40]. This peptide (ILHNGAYSL) bound to HLA-A2.1 with intermediate affinity (IC50 74.6 nM). The HER-2(9_{435}) epitope was tested using an *in vitro* immunization protocol and found to elicit CTLs that killed peptide-sensitized target cells. The CTLs elicited also recognized the HER-2/neu antigens, as it specifically killed tumor cells expressing the HLA-A2.1 and HER-2/neu antigens (see below in preliminary data). Furthermore, recognition of the tumor cell targets was significantly inhibited by unlabeled (cold) targets pulsed with HER-2(9_{435}), but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (see below). Thus, the CTLs induced by HER-2(9_{435}) are antigen specific.

A potential limiting factor for peptide based immunotherapy is related to a defined antigenic repertoire which is HLA restricted. This factor, inherent to all peptide-based approaches, restricts patient access. Additionally, because individual peptides only have the potential to induce epitope-specific CTL, the vast majority of potential tumor antigens are not targeted. In this setting, tumor down regulation of individual antigens or HLA epitopes promotes immune evasion. Recent evidence, however, suggests that this problem of epitope restriction may not be as physiologically important as was previously postulated. Specifically, it has now been clearly demonstrated that a T cell response induced against one epitope can stimulate CTL response to other target epitopes through a mechanism termed epitope spreading [3, 41, 42]. Using an experimental autoimmune encephalitis model, Vanderlugt et al. have demonstrated that disease progression is associated with the development of epitope-specific helper T cells, which are distinct from those initiating the disease. Transfer of secondary $CD4^+$ cells to naïve mice induces the disease phenotype and the disease is abrogated by blocking the secondary T cell response even though the primary T cell response remains intact [43, 44]. Disis demonstrated epitope spreading in 84% of patients vaccinated with HER-2/neu peptides, reflecting the initiation of an endogenous immune response. The immunity persisted after active immunizations ended [3]. These data suggest that peptide based approaches to cancer immunotherapy may indirectly stimulate multiple tumor reactive CTL against minor antigens in

the presence of residual tumor. Based on this concept, the current study is designed as a therapeutic approach, with peptide epitope selection designed to enhance the number of potential candidates.

In addition to class I epitopes, immunogenic HLA-DR restricted class II epitopes have been defined for HER-2/neu. $CD4^+$ helper T lymphocytes (T_H) responses play an essential role in immunologically mediated anti-tumor immunity [45]. T_H lymphocytes provide CTLs with growth-stimulating cytokines, prime/activate DCs to effectively present antigen to naive CTL precursors [46-48] and they are important in the development of immune memory [49-51]. The development of IgG antibodies to HER-2/neu and the identification of $CD4^+$ T cells that secrete cytokines in response to HER-2/neu peptides or recombinant HER-2/neu protein suggest responses to helper T cells [52-57]. A promiscuous MHC class II T_H epitope has been identified for the HER-2/neu antigen ($HER\text{-}2_{883}$). T cell responses are restricted by HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53 [58]. Peptide-induced T cells were effective in recognizing naturally processed HER-2/neu protein. The peptide $HER\text{-}2_{883}$, (KVPIKWMALESILRRRF), which was selected by computer algorithm, was tested for its capacity to stimulate $CD4^+$ T cells isolated from four healthy, MHC-typed individuals (DR1/11, DR1/13, DR4/15, DR7/17) in primary *in vitro* culture using peptide pulsed autologous DCs. T cells that proliferated were found to react with peptide and recombinant HER-2/neu intracellular domain protein presented by autologous DCs (see below). These results, showing reactivity with recombinant protein, suggest that $HER\text{-}2_{883}$ is naturally processed, as the peptide stimulated T cells react with DCs primed with recombinant protein. Clearly, $HER\text{-}2_{883}$ is a naturally processed peptide epitope and is promiscuous for multiple HLA-DR epitopes, making it an ideal candidate for therapeutic applications.

1.7 Because of the expression of MUC1 and HER-2/neu in multiple cancers, the development of this peptide-based immunotherapy can potentially impact the treatment of multiple disease entities, not only adenocarcinomas but hematopoietic malignancies as well. There is considerable interest in the use of the MUC1 peptide vaccination for treatment of multiple myeloma following transplant when there is minimal residual disease prior to remission.

1.8 GM-CSF

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a commercially available cytokine currently used in patients undergoing chemotherapy to shorten the duration of post-chemotherapy neutropenia. Recently published evidence also suggests that GM-CSF may play a role as an immune adjuvant [59, 60]. The following observations illustrate the mechanisms by which GM-CSF can potentiate the immunogenicity of an antigen: 1) GM-CSF is a key mediator of dendritic cell (DC) maturation and function [61]; 2) GM-CSF increases surface expression of class I and II MHC molecules as well as co-stimulatory molecules of dendritic cells *in vitro* [61]; 3) GM-CSF enhances antibody responses to known immunogens *in vivo* [62]; 4) tumor cells transfected with genes encoding/expressing GM-CSF are able to induce long lasting, specific anti-tumor immune responses *in vivo* [63]; 5) GM-CSF encapsulated in biodegradable microspheres mixed with whole tumor cells resulted in systemic anti-tumor immune responses comparable to those of GM-CSF transfected tumor cells [64]. Therefore, addition of GM-CSF to an oligopeptide antigen may substantially enhance its immunogenicity.

In an attempt to optimally enhance the immunogenicity of the peptides we will deliver the antigens and GM-CSF emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA-51). This delivery mechanism should be comparable to a previously demonstrated delivery mechanism utilizing GM-CSF suspended in microspheres and mixed with tumor cells (antigens). We hypothesize that the emulsified GM-CSF in close proximity to tumor antigen peptides will substantially enhance their immunogenicity. This proximity of antigen and GM-CSF seems to be necessary for the adjuvant effect of GM-CSF, as systemic administration of

equivalent doses in animal models has not demonstrated adjuvant activity. Also, the adjuvant/local inflammatory properties of IFA may play a role in attracting antigen presenting cells to the site of injection [53]. We have preliminary data demonstrating the plausibility of such a mechanism.

1.9a Preliminary data demonstrating the feasibility of this approach already exists. Rosenberg and investigators published effective generation of peptide-specific T cells in melanoma patients immunized with peptides derived from gp100 [65]. Despite the demonstration of a specific immune response, no clinical responses were detected. Addition of systemic GM-CSF resulted in more pronounced CTL and delayed type hypersensitivity reactions and in a few cases objective tumor regressions. Salgaller et al. utilized a peptide derived from the gp100 epitope suspended in IFA and demonstrated generation of specific T cell responses to the peptide in melanoma patients [66]. Both studies suggest that increased immunogenicity of the peptide antigens leads to a more pronounced T cell response, which in some cases results in a clinically relevant anti-tumor effect. In the proposed study, we will combine the immunoadjuvant effects of both IFA and GM-CSF with the goal of increasing the immunogenicity of the MUC1 and HER-2/neu immunodominant peptides.

Preliminary observations in an ongoing clinical study (MC9973) utilizing HLA-A2 specific melanoma differentiation antigen peptide vaccines in which the peptide is emulsified in a suspension of IFA and GM-CSF is demonstrating enhanced skin reactions if peptide emulsified in IFA is administered in the presence of GM-CSF. A dose of 50 μg of GM-CSF in the presence of IFA and peptide results in extensive local skin reactions as well as evidence of a clinical response in one of seven patients thus far. No changes in the numbers of peptide specific CTLs were observed. However, a recent publication demonstrated superior numbers of vaccine specific CTLs generated in a peptide vaccine utilizing 225ug of GM-CSF in IFA) [67]. This would suggest a dose/response relationship of GM-CSF and anti-peptide vaccine CTL frequencies as determined by ELISPOT and tetramer assays. Therefore, in the current trial we propose to use 225ug of GM-CSF suspended in IFA (montanide ISA-51).

1.9b CpG

Therapeutic properties of bacteria in the treatment of malignant diseases (i.e. Coley's toxin) is an observation that has permeated the oncology literature for almost a century. More recently, it has been demonstrated that bacterial DNA possesses unique immunomodulatory features of potential utility in cancer therapy. Specifically, unmethylated CpG are able to stimulate NK cells and B cells. Furthermore, synthetic oligodeoxynucleotide (ODN) constructs containing unmethylated CpG motifs (CpG-ODN) were able to activate dendritic cells (DC) enhancing their antigen processing/presentation properties and stimulating production of Th1 cytokines necessary for CTL immune responses. Thus, CpG ODN appeared to function as an immune adjuvant. Several preclinical and clinical works illustrate the ability of CpG-ODN to function as a potent immune adjuvant for various forms of vaccines. One of the more interesting works, pertinent to this study, demonstrates the ability of CpG ODN to induce CTLs against a peptide vaccine when administered in conjunction with incomplete Freund's adjuvant (IFA) [68]. These authors used a MART-1/Melan-A_{26-35} peptide emulsified in IFA with or without the addition of 50ug of CpG ODN to immunize human D^b (HHD) A2 transgenic mice. Their data suggest superior anti-peptide immunization in the CpG-ODN immunized group as determined by the frequency of tetramer positive CTLs. Our own data support these findings demonstrating superior immunization efficacy of IFA+CpG-ODN with ova peptide of C57BL/6 mice when compared to either IFA+peptide or complete Freund's adjuvant (CFA) + peptide (data not shown). An additional benefit to the CpG-ODN adjuvant is that it has been shown to be especially good at enhancing cellular and humoral immunity and promoting a Th1-type of response in older mice [69]. The population that develops cancer is mainly older individuals, thus the CpG-ODN adjuvant may be particularly relevant for this trial. Based on preclinical data

suggesting the potent immune adjuvant properties of CpG co-emulsified with peptides in IFA, we elected to test the efficacy of CpG-ODN in the setting of a peptide vaccine immunization in this clinical trial. The dose of CpG-ODN that we decided to use in this study is 2mg/vaccine. The dose is based on published data demonstrating a direct dose-dependent relationship of CpG-ODN (0.125 –1.0 mg) and magnitude of measured immune responses (HepB vaccine adjuvant [70]). This is well below the highest tested doses of 20mg/week. Based on these observations we feel that the 2mg dose is a reasonable starting point for a CpG-ODN adjuvant suspended in Montanide ISA 51 alone or in combination with GM-CSF.

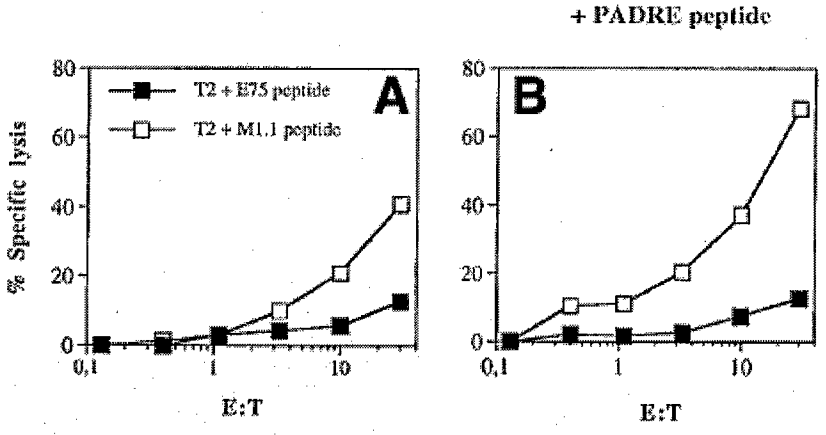
1.9c Preliminary Data

Preliminary data will be presented in multiple sections. First, we will provide data to support the choice of MUC1 and HER-2/erbB2 antigenic epitopes for this trial. Next, we will define our experience using peptides to stimulate tumor reactive T cells for cancer immunotherapy. Finally, we will discuss our experience with the immune adjuvants GM-CS and CpG-ODN. These preliminary data provide a strong foundation for the current proposal.

1.9c1 Identification of CTL Epitopes from MUC1

Using a computer analysis of the MUC1 amino acid sequence, two novel peptides were identified with a high binding probability to the HLA-A2 molecule [33]. Two peptides from MUC1 were identified; one from the tandem repeat M1.1 ($STAPPVHNV_{950-958}$) and one from the leader sequence M1.2 ($LLLLTVLTV_{12-20}$). The presence of the V in position 6 increases the binding of the M1.1 peptide to the HLA-A2 molecule. There is some variability in the tandem repeats in MUC1 and this sequence is found in the last tandem repeat. Cytotoxic T cells were induced from healthy donors by primary *in vitro* immunization using peptide-pulsed dendritic cells. The peptide-induced CTL lysed tumors endogenously expressing MUC1 in an antigen-specific and HLA-A2-restricted fashion.

CTL.M1.1



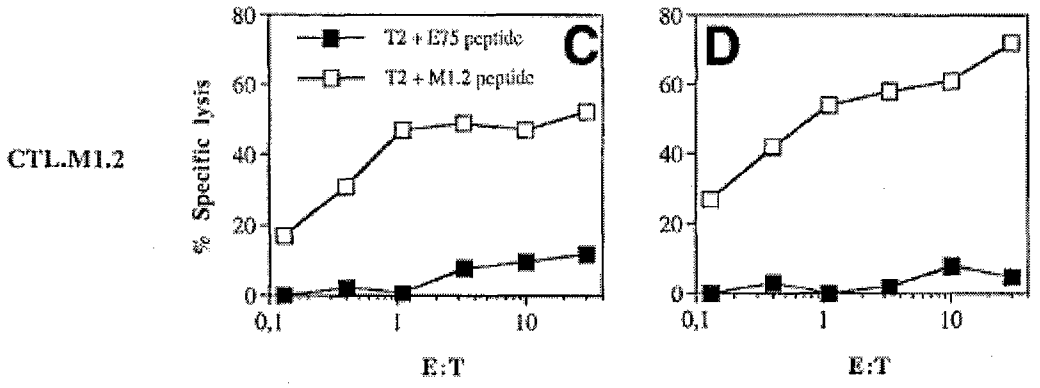


Figure 1. *Induction of CTL responses by peptide-pulsed dendritic cells. Adherent peripheral blood mononuclear cells were grown for 7 days with GM-CSF, IL-4, and TNF alpha. DCs pulsed with the synthetic peptides derived from the MUC1 protein (M1.1 and M 1.2) were used to induce a CTL response in vitro. In addition to the MUC1 peptide DCs were incubated with the PAN-DR binding peptide PADRE as a T-helper epitope. Cytotoxic activity of induced CTL was determined in a standard ^{51}Cr-release assay using T2 cells as targets pulsed for 2 hours with 50 μg of the cognate (open symbols) or irrelevant HER-2/neu protein-derived protein derived E75 peptide (solid symbols). (data reproduced from Brossart 1999 [33])*

Next, the ability of the induced MUC1-specific CTL lines to lyse tumors expressing MUC1 was tested. MCF-7 cells that express MUC1 endogenously and are HLA-A2 positive were used as targets in a standard ^{51}Cr-release assay. The controls were SK-OV-3 cells, which express MUC1, but are HLA-A2 negative and the immortalized B cell line, Croft, which is A2 positive and was pulsed with MUC1 M1.1 or M1.2 peptides or the irrelevant HER-2/neu E75 peptide.

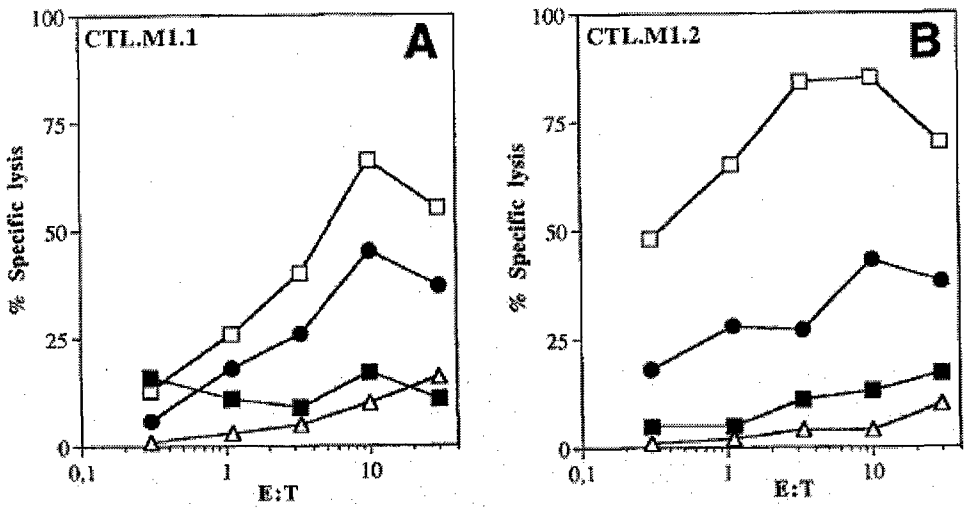


Figure 2. *Lysis of cancer cells endogenously expressing MUC1 by CTL.M1.1 (A) and CTL.M1.2 (B). Human breast cancer cell line MCF-7 (HLA-A2$^+$/MUC1$^+$), ovarian cancer cell line SK-OV-3 (HLA-A2$^-$ /MUC1$^+$), and the immortalized B-cell line Croft (HLA-A2$^+$/MUC1$^-$) were used as targets in a standard ^{51}Cr-release assay. Croft cells were pulsed with the MUC1 peptides or an irrelevant HER-2/neu-derived peptide E75. (■) Croft + E75 peptide; (□) Croft + M1.1 (A) or M1.2 (B); (•)MCF-7; (Δ) SK-OV-3.*

We have chosen to use the M1.1 peptide based on the large amount of data on the response to the MUC1 tandem repeat peptide, both in the human situation as well as in the mouse. Obviously only the human data are relevant for the clinical trials. We will use a HER-2/neu helper epitope (see below, not the PADRE helper epitope)

In the case of HER-2/neu, we have identified a novel CTL epitope HER-2 (9_{435}), which bound HLA-A2.1 with intermediate affinity (IC_{50} 74.6 nM). The peptide identified is: ILHNGAYSL. The .221(A2.1) cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human lymphoblastoid cell line .221, was used as target (peptide loaded) to measure activity of HLA-A2.1 restricted CTL [71]. The CTLs elicited following in vitro stimulation effectively killed HLA-A2.1$^+$ tumor cells, showing that the antigen is appropriately processed by tumors (Fig. 3A). In addition, recognition of the tumor cell target was significantly inhibited by unlabeled (cold) target pulsed with HER-2 (9_{435}) peptide, but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (Fig. 3B).

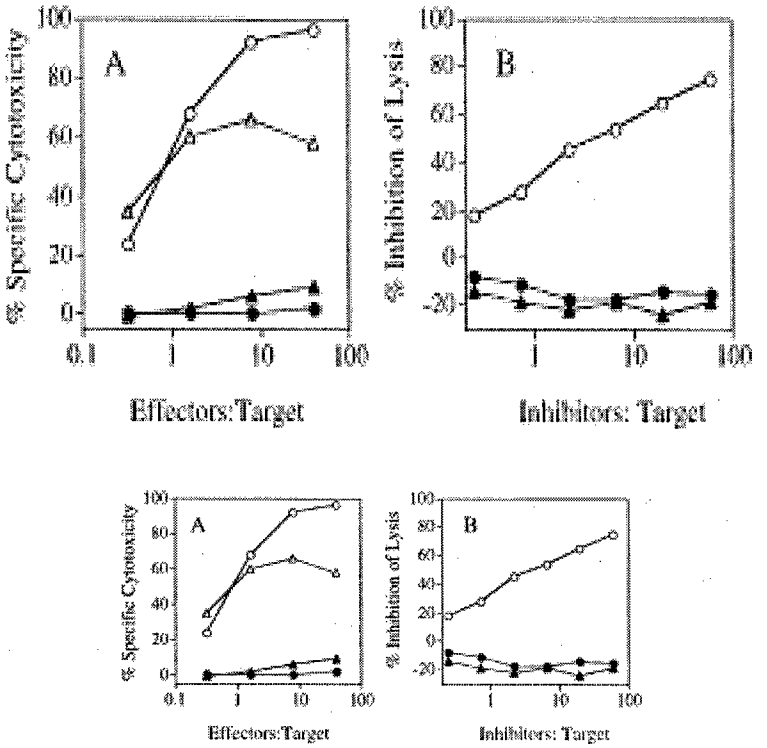


Figure 3. *HER-2(9_{435}) specific CTL can kill tumor cells. (A) HER-2(9_{435}) specific CTL were used as effector cells to test for the lysis of the following target cell lines: o, .221A2.1 pulsed with HER-2(9_{435}); ●, .221A2.1 without peptide; Δ, SW403 (colon CA, A2$^+$, HER-2/neu$^+$); ▲, HT-29 (colon ca, A2$^-$, HER-2/neu$^+$). (B): Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr labeled SW403 cells at an effectors/target ratio of 10:1 by the HER-2(9_{435}) specific CTL was blocked at various Inhibitors/Target ratios by the following cold targets: o, .221A2.1 pulsed with HER-2(9_{435}); ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); ●, .221A2.1 without peptide.*

In addition to the class I epitopes described above, a promiscuous MHC class II epitope was defined for HER-2/neu using the algorithm tables published by Southwood et al. [58, 72]. The epitope identified is HER-2_{883} (KVPIKWMALESILRRRF). It is important to show that these peptides represent true T cell epitopes that are relevant for the development of tumor immunotherapy. For these experiments autologous PBMCs or DCs were used as APCs and recombinant DNA derived intracellular domain or extracellular domain protein fragments of HER-2/neu were used as a source of antigen. The data in Fig. 4 show that four HER-2_{883}-reactive T cell lines proliferated well to HER-2/neu intracellular domain protein, which encompasses the HER-2_{883} peptide but not to HER-2/neu extracellular domain (ECD), which lacks HER-2_{883}.

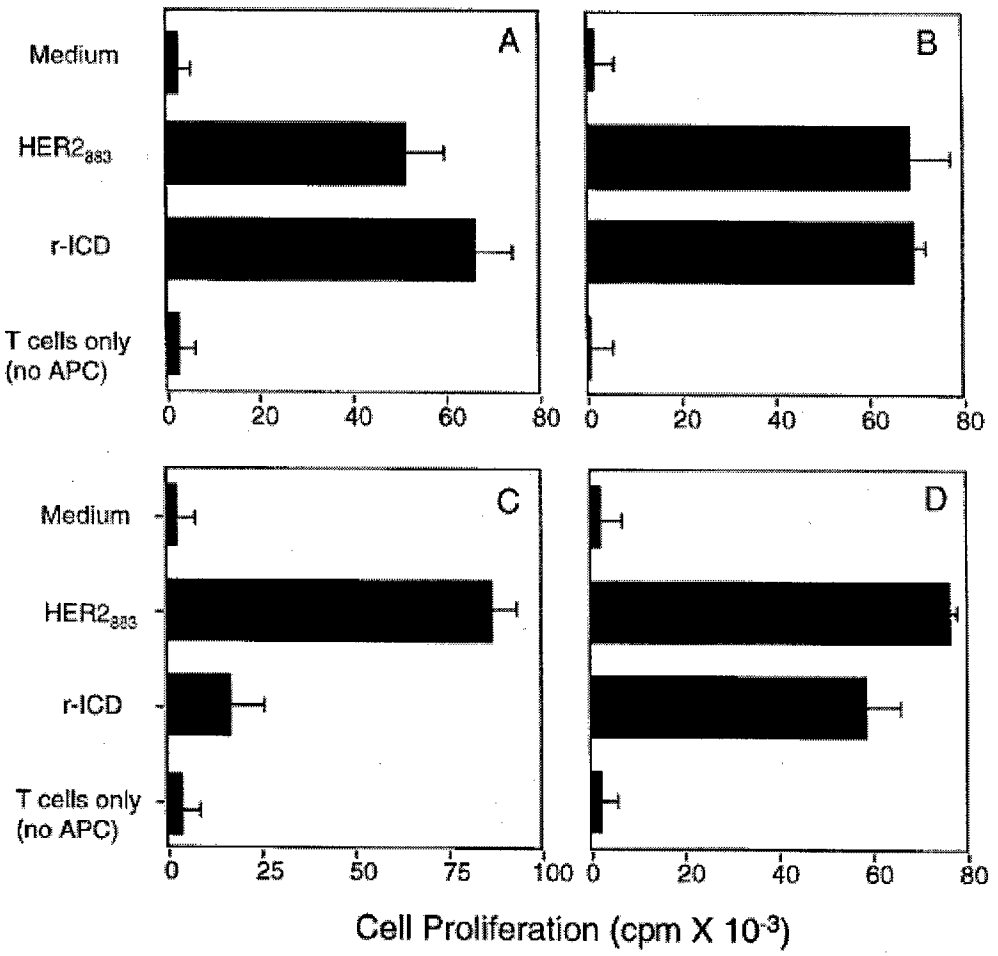


Figure 4. *HER-2_{883}-specific $CD4^+$ T cells can recognize recombinant HER-2/neu intracellular domain (r-ICD) protein presented by autologous Dcs in the context of several HLA-DR alleles. The HER-2_{883}-reactive HTLs, TCL-7C (panel A, HLA-DR53 restricted), TCL-6D (panel B, HLA-DR4-restricted), a clone of TCL-1D (panel C, HLA-DR52-restricted), and TCL-1E (panel D. HLA-DR53 restricted), were tested for their capacity to proliferate to autologous DCs in the presence of HER-2_{883} peptide (2.5 mg/ml) or recombinant HER-2/neu recombinant ICD protein (10 mg/ml). No significant proliferative response was observed against HER-2/neu ECD protein (data not shown). Values shown are the means of triplicate determinations; bars, SD.*

1.9d Justification of vaccination strategy

1.9d.1 **Peptide dose** (1000ug): Over the last several years there has been extensive debate over the optimal dose of peptide in a variety of peptide immunization cancer clinical trials. Peptide doses have ranged from 50ug to 2500ug in various studies. Currently, the largest peptide vaccine clinical trial (E4697) utilizes a peptide dose of 1000ug. There are several published studies evaluating peptide vaccine dose-responses [66, 73], suggesting that 1000ug of peptide would be a reasonable vaccine dose for phase I/II clinical testing.

1.9d.2 **GM-CSF suspended in Montanide ISA 51 as a vaccine adjuvant.** The utility of GM-CSF suspended in montanide ISA 51 as an effective vaccine adjuvant has already been demonstrated in pre-clinical and clinical studies. Our own pre-clinical data (Fig 5) demonstrates a bell shaped dose-response curve for GM-CSF co-emulsified with 10ug

of *ova* peptide in montanide ISA 51. Two weeks after immunization, the optimal dose of GM-CSF in the mouse model appears to be 100ug. In humans, Slingluff et al. demonstrated successful peptide immunization using 225ug of GM-CSF suspended in montanide ISA-51[67]. Up to 80% of treated patients demonstrated effective immunization with melanoma differentiation antigen peptides. Our clinical data using 10, 50, 75 and 100 ug of GM-CSF suspended with peptides in Montanide ISA-51 failed to demonstrate effective generation of anti-peptide CTLs. In view of these data, we felt that it was reasonable to utilize the same dose of GM-CSF used by Slingluff [67] (225ug) with our current set of peptides. If successful, further studies will be performed attempting to generate a dose-response curve of GM-CSF and immunization efficacy similar to that of the mouse model.

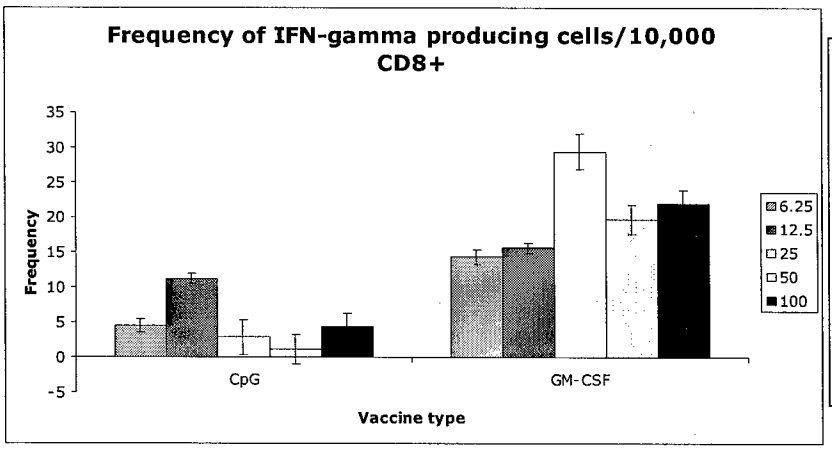


Fig. 5: C57BL6 mice (3 per group) were immunized with 10ug of ova peptide suspended in Montanide ISA51 and varying concentrations of CpG or GM-CSF. Represented are the frequencies of ova specific CTLs (IFN gamma ELISPOT) isolated from splenocytes on day 12 post immunization. Similar dose/response curves were observed in two other experiments.

1.9d.3 **CpG suspended in Montanide ISA 51 as vaccine adjuvant.** As described in section 15.7, the co-emulsification of peptide antigens with CpG and Montanide ISA-51 is an effective means of generation of peptide specific CTLs in a pre-clinical model. Our own data confirm these findings using non-transgenic mice immunized with ova peptide co-suspended with CpG in Montanide ISA 51 (Fig. 5). The dose of CpG used in the current study was empirically selected based on the results of a phase I clinical trial utilizing CpG (abbreviated as ISS in Fig. 6 legend) as an immune adjuvant for hepatitis B vaccine immunization in healthy volunteers. In this study, volunteers were immunized with an intramuscular injection of hepatitis B vaccine (20ug) mixed with CpG in one of the following concentrations: 225ug, 650ug, 1000ug or 2250ug. A booster injection was administered 2 months later. Serologic data demonstrated (Fig 6) maximal immunization efficacy at CpG doses between 1000 and 2250ug. Based on these data suggesting a bell-shaped dose response curve for CpG (optimum may be between doses 1000ug and 2250ug) as well as our pre-clinical bell-shaped dose response curve, we elected to proceed with a CpG dose of 2000 ug.

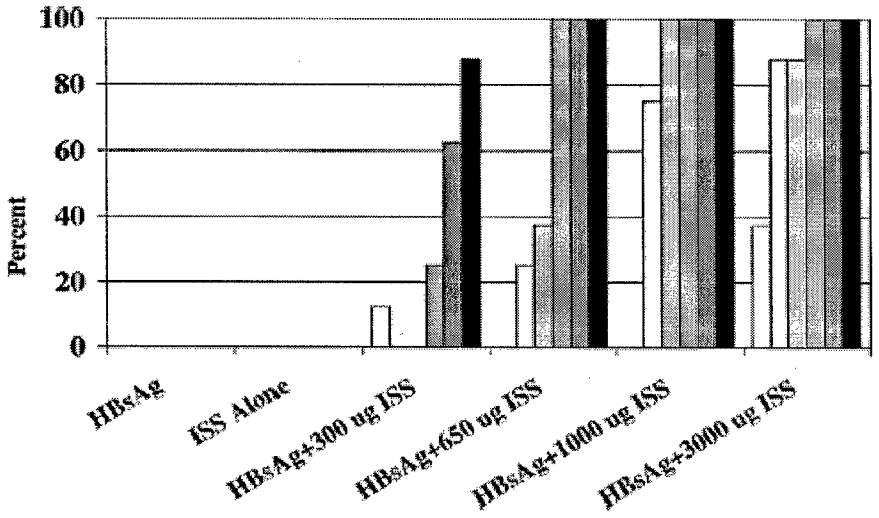


Figure 6: Proportion of participants achieving a protective antibody level (≥10mIU/mL) at various time points after immunization. Time points are (by increasing darkness of bar shade) 7 days after dose 1, 28 days after dose 1, 56 days after dose 1, 7 days after dose 2, 4 months after dose 2. CpG is designated as ISS.

The target population for this clinical trial, to whom the study findings will be generalized, are patients with a history of completely treated stage II or III breast adenocarcinoma that is MUC1 positive, currently off active therapy (with the exception of hormonal therapy) with no evidence of tumor relapse.

2.0 Goals

2.1 Primary Goal

To determine the safety and immunization efficacy of MUC1 and HER-2/neu peptide vaccines combined with CpG, GM-CSF or both, as immune adjuvants suspended in Montanide ISA-51.

2.2 Secondary Goal

To describe the impact of immunization on clinical outcomes in patients with MUC1 positive breast cancer.

3.0 Patient Eligibility

3.1 Inclusion criteria

3.11 Age ≥18 years.

3.12 Completed "standard first line therapy ONLY" (including adjuvant therapy) for breast cancer, clinical stage II and III (≥3 months prior to registration) and currently with no evidence of disease. Current use of "anti-estrogen" therapy is allowed.

3.13 Histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy.

3.15 MUC1 positive breast cancer by central review.

3.16 HLA-A2 positive.

3.17 The following laboratory values obtained ≤14 days prior to registration:

- Hemoglobin ≥8.0 g/dL
- Platelets ≥75,000/uL
- ANC ≥1,500/uL
- Creatinine ≤2 x ULN
- AST ≤ 2 x ULN

3.18 Capable of understanding the investigational nature, potential risks and benefits of the study and capable of providing valid informed consent.

3.19a Willingness to return to Mayo Clinic Rochester, Scottsdale, or Jacksonville for treatment and study-related follow up. Study treatment will be administered only at the Mayo Clinic site where the patient was enrolled. Post-treatment study follow-up is allowed at the other participating Mayo Clinic sites.

3.19b Willingness to provide the blood and tumor specimens and complete the imaging studies as required by the protocol.

Note: The goals of this study include assessment of the biologic effects on surrogate markers of the agent(s) being tested and are, therefore, contingent upon availability of the blood and tumor specimens and completion of the required imaging studies.

3.2 Exclusion criteria

3.21 ECOG performance status (PS) 3 or 4 (see Appendix I).

3.22 Uncontrolled infection.

3.23 Any of the following:
- Known HIV infection
- Other circumstances (i.e. concurrent use of systemic immunosuppressants and immunocompromising condition) that in the opinion of the physician renders the patient a poor candidate for this trial

3.24 Failure to fully recover from acute, reversible effects of prior breast cancer therapy regardless of interval since last treatment.

3.25 Any of the following:
- Pregnant women
- Nursing women unwilling to stop breast feeding
- Women of childbearing potential who are unwilling to employ adequate contraception (diaphragm, birth control pills, injections, intrauterine device [IUD], or abstinence, etc.)

NOTE: This study involves an investigational agent whose genotoxic,mutagenic and teratogenic effects on the developing fetus and newborn are unknown.

3.26 Other concurrent chemotherapy, immunotherapy, radiotherapy, or any ancillary therapy considered investigational (utilized for a non-FDA-approved indication and in the context of a research investigation).

3.27 Radiographic evidence of disease at the time of enrollment.

3.28 Any prior invasive malignancies ≤5 years (with the exception of curatively-treated basal cell or squamous cell carcinoma of the skin or carcinoma in situ of the cervix).

3.29 Primary surgery for breast cancer **beyond 3 years** at time of registration.

4.0 Test Schedule

Tests and procedures	≤14 days prior to registration	Prior to each subsequent treatment (q 4 weeks)	At 4 weeks after last treatment	Observation q 3 months for 2 years
History and assessment, wt, PS	X	X^R	X	X
Height	X			
Hematology group: WBC, ANC, Hgb, PLT	X^R	X^8	X^R	
Chemistry group: total and direct bilirubin, AST, creatinine.	X^R	X^8	X^R	
HLA class I and II typing [R]	At any time prior to registration			
Serum pregnancy test[1]	X			
Tumor typing[5R]	At any time prior to registration			
Tumor evaluation by imaging study (x-ray, CT or PET)	X			X^2
DTH skin testing (common recall antigens)[3, R]	X	Prior to cycle 6 only		
Research blood specimens[7] See section 14.0	X	X^4		X^4
Acute toxicity evaluation[6]		X	X	

1. For women of childbearing potential, must be obtained ≤7 days prior to registration.
2. Imaging will be performed per "standard of care" for patients and at the discretion of the treating physician
3. DTH skin testing will be performed using the same complement of antigens in routine use at the treatment site.
4. Research blood samples will be performed before registration as well as prior to **cycles 3, 5 and 6** of therapy as well as every other cycle of long-term follow-up **starting at 3 months** after completion of Rx up to two years.
5. Tumor tissues will be stained for MUC-1 and HER-2/neu.
6. Acute toxicity evaluations (physical exam and laboratory testing) will be performed for the purpose of evaluating potential immediate side effects of immunization.
7. Research blood specimens will be collected only if serum hemoglobin for the given collection is ≥10 g/dL. If hemoglobin is <10 g/dL, research blood samples will be postponed until the next study office visit.
8. Research funded prior to cycles 2 and 5.

R. Research Funded

5.0 Stratification Factors

Her-2/neu status: Positive vs. negative vs. unknown.

6.0 Registration/Randomization Procedures

6.1 To register a patient, access the Mayo Clinic Cancer Center (MCCC) web page and enter the remote registration/randomization application. The remote registration/randomization application is available 24 hours a day, 7 days a week. Back up and/or system support contact information is available on the Web site. If unable to access the Web site, call the MCCC Registration/Randomization Center at (507) 284-2753 between the hours of 8 a.m. and 5:00 p.m. Central Time (Monday through Friday).

The instructions for remote registration are available on the MCCC web page (http://hsrwww.mayo.edu/ccs/training) and detail the process for completing and confirming patient registration. Prior to initiation of protocol treatment, this process must be completed in its entirety and a MCCC subject ID number must be available as noted in the instructions. It is the responsibility of the individual registering the patient to confirm the process has been successfully completed prior to release of the study agent. Patient registration via the remote system can be confirmed in any of the following ways:

- Contact the MCCC Registration/Randomization Center (507) 284-2753. If the patient was fully registered, the Registration/Randomization Center staff can access the information from the centralized database and confirm the registration.
- Refer to "Instructions for Remote Registration" in section "Finding/Displaying Information about A Registered Subject."

6.2 A mandatory translational research component is part of this study. The patient will be automatically registered onto this component (Section *14.0)*.

6.3 A signed HHS 310 form must be on file in the Randomization Center before an investigator may register any patients. Ongoing approval documentation must be submitted (no less than annually) to the Randomization Center.

6.4 Prior to accepting the registration/randomization, the remote registration/randomization application will verify the following:

- IRB approval at the registering institution
- Patient eligibility
- Existence of a signed consent form
- Existence of a signed authorization for use and disclosure of protected health information

6.5 Treatment on this protocol must commence at Mayo Clinic Rochester, Scottsdale or Jacksonville under the supervision of a medical oncologist or hematologist.

6.6 Treatment cannot begin prior to registration and must begin $\leq$7 days after registration.

6.7 Pretreatment tests/procedures must be completed within the guidelines specified on the test schedule.

6.8 All required baseline symptoms must be documented and graded.

6.9a Study drug availability checked.

7.0 Protocol Treatment

7.1 For the purposes of this trial, patients will be recruited from the breast cancer practice of the Mayo Clinic Cancer Center. Patients who are undergoing regular follow-up visits by Mayo Clinic oncologists, are interested in this study, and fulfill all eligibility criteria will be offered enrollment. The patients' primary physicians, co-investigators in this study, will have the opportunity to offer the study to interest patients during their regularly scheduled follow-up visits. It is not expected that recruitment or advertisement materials will be used.

The patients who are enrolled will be assigned a 'study number' which will be used for their identification, and that of their data, throughout their participation in the clinical trial.

The Informed Consent process will take place during the patient's regular follow-up visits with their oncologists, co-investigators in the clinical trial. The informed consent interview will begin as part of the patient's regular follow-up visit. At that time, interested patients will be given information about the study, and if interested, will also receive a copy of the Informed Consent document. Patients will have the opportunity to discuss the details of the study during this visit or, more likely, will be given the consent form and offered to review the document at home and schedule a follow-up visit if they are interested in taking part on the study. This way the patients will have a chance to investigate and discuss the study on their own. If interested, the patients will set-up a 2nd visit with their oncologists specifically for the purpose of deciding on study participation. At that visit, all issues of concern for the patient will be addressed, eligibility reviewed and, if appropriate, the Consent Form will be signed.

7.2 As part of the registration process described in Section 6.0, the Mayo Clinic Cancer Center (MCCC) Remote Registration application will assign patients to arms A through C.

7.3 Treatment Schedules:

Arm A

	Agent	Dose	Route	RxDays	ReRx
Arm A	Montanide ISA-51	1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28-32 days) x 6 cycles
	MUC1 (STAPPVHNV)	1000ug			
	HER-2 peptide 1 (ILHNGAYSL)	1000ug			
	HER-2 peptide 2 (KVPIKWMALESIL)	1000ug			
	GM-CSF	225ug			

Arm B

	Agent	Dose	Route	RxDays	ReRx
Arm B	Montanide ISA-51	1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1 (STAPPVHNV)	1000ug			
	HER-2 peptide-1 (ILHNGAYSL)	1000ug			
	HER-2 peptide-2 (KVPIKWMALESILRRRF)	1000ug			
	GpG	2mg			

Arm C

	Agent	Dose	Route	RxDays	ReRx
Arm C	Montanide ISA-51	1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1 (STAPPVHNV)	1000ug			
	HER-2 peptide-1 (ILHNGAYSL)	1000ug			
	HER-2 peptide-2 (KVPIKWMALESILRRRF)	1000ug			
	GM-CSF	225ug			
	CpG	2mg			

7.4 Fifteen patients per arm (total of 45) will be randomly assigned to receive one of the three treatment schedules. Doses will not be escalated in any individual patient. It is not anticipated that there will be toxicity experienced with these regimens.

Vaccines will be prepared in a single vial and administered as multiple (2-3) subcutaneous injections in regions of un-disturbed axillary or inguinal lymph nodes. Each vaccine cycle will be administered into a single lymph node draining area. Subsequent vaccination cycles will be administered to other (rotating) undisturbed lymph node drainage sites.

The risks to the patients taking part on this study are minimal. The main risks are those of an allergic reaction to the components of the peptide vaccine (local or systemic). To minimize risk, patients will be observed by a registered nurse for 30 minutes following each immunization. On-site physicians will be available in the unlikely event that complications do occur. Risks due to phlebotomy will be minimized by ensuring that all patients will undergo phlebotomy by certified phlebotomists. All patients will be provided detailed contact information so that they are able to contact their treating physicians/co-investigators if they experience problems (medical or otherwise) while undergoing therapy in this study.

There are no antidotes available for the peptide vaccines used in this protocol. If patients develop symptoms as a result of the vaccines (e.g. allergic reactions), those patients will be treated accordingly.

The benefit to patients who undergo treatment in this study is unknown.

As IND sponsor, the Principal Investigator will monitor the protocol in accordance with 21 CFR 312, as indicated in portions of section 4.0, the test schedule; section 10.0, the adverse event reporting; and section 15, the drug information.

8.0 Dosage Modification Based on Adverse Events - Adjustments are based on adverse events observed since the prior dose.

ALERT: *ADR reporting may be required for some adverse events (See Section 10)*

→ → *Use Common Terminology Criteria for Adverse Events (CTCAE) v3.0 unless otherwise specified* ← ←			
CTCAE CATEGORY	ADVERSE EVENT	AGENT	DOSAGE CHANGE OR OTHER ACTION
AT TIME OF RETREATMENT			
ALLERGY/ IMMUNOLOGY	≥Grade 2 allergic reaction/ hypersensitivity	Montanide	Discontinue vaccinations indefinitely and begin event monitoring.
	≥Grade 2 autoimmune reaction (excluding vitiligo)	GM-CSF	Discontinue vaccinations indefinitely and begin event monitoring.
ALL OTHERS	≥Grade 3 Hematologic or ≥Grade 3 Nonhematologic (excluding alopecia) ≥Grade 2 neurologic	CpG Peptides	Discontinue vaccinations indefinitely and begin event monitoring.

9.0 Ancillary Treatment/Supportive Care

9.1 Patients should receive blood product support, antibiotic treatment and treatment of other newly diagnosed or concurrent medical conditions.

9.2 Patients participating in this clinical trial are not to be considered for enrollment in any other study involving a pharmacologic agent (drugs, biologics, immunotherapy approaches, gene therapy) whether for symptom control or therapeutic intent.

10.0 Adverse Event (AE) Reporting and Monitoring

10.1 This study will utilize the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 for adverse event monitoring and reporting. The CTCAE v3.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov/CTC3/ctc_ind_term.htm). All appropriate treatment areas should have access to a copy of the CTCAE v3.0.

10.11 Adverse event monitoring and reporting is a routine part of every clinical trial. First, identify and grade the severity of the event using the CTCAE. Next, determine whether the event is expected or unexpected (refer to Section 15.0 and/or product literature) and if the adverse event is related to the medical treatment or procedure (see Section 10.12). With this information, determine whether an adverse event should be reported as an expedited report (see Section 10.2) or as part of the routinely reported clinical data.

Expedited adverse event reporting requires submission of a written report, but may also involve telephone notifications. Telephone and written reports are to be completed within the timeframes specified in Section 10.2. All expedited adverse event reports should also be submitted to the local Institutional Review Board (IRB).

10.12 Assessment of Attribution

When assessing whether an adverse event is related to a medical treatment or procedure, the following attribution categories are utilized:

Definite - The adverse event *is clearly related* to the investigational agent(s).
Probable - The adverse event *is likely related* to the investigational agent(s).
Possible - The adverse event *may be related* to the investigational agent(s).
Unlikely - The adverse event *is doubtfully related* to the investigational agent(s).
Unrelated - The adverse event *is clearly NOT related* to the investigational agent(s)

10.2 Expedited Adverse Event Reporting Requirements

Phase I, II and III Studies (Investigational)

	Grade 4 or 5[1] Unexpected with Attribution of Possible, Probable, or Definite	Other Grade 4 or 5 or Any hospitalization during treatment[6]	Secondary AML/MDS[2]
Notify the Cancer Center IND Coordinator[3] within 24 hours	X		
Submit written report within 5 working days[4]	X		
NCI/CTEP Secondary AML/MDS Report Form within 15 working days[5]			X
Submit Grade 4 or 5 Non-AER Reportable Events/Hospitalization Form within 5 working days.[6]		**X**[6]	

1. Includes all deaths within 30 days of the last dose of investigational agent regardless of attribution or any death attributed to the agent(s) (possible, probable, or definite) regardless of timeframe.
2. Reporting for this AE required during or after treatment.
3. Notify the Cancer Center IND Coordinator (Mayo Clinic - Rochester) by telephone (507) 284-0938 and/or submit a written event summary via fax to (507) 538-7164.
4. Use *Adverse Event Expedited Report – Single Agent* or *Multiple Agents* report form. Submit to the Cancer Center IND Coordinator (Mayo Clinic - Rochester) and to the Cancer Center Protocol Development Coordinator (PDC) for IRB reporting. The IND Coordinator will review the event in consultation with the IND holder and report to the Food and Drug Administration (FDA) as warranted by the event and required by U.S. federal regulations.
5. Submit per form-specified instructions and provide copy to Cancer Center IND Coordinator for review and FDA reporting (as warranted by the event) and the Cancer Center PDC for IRB reporting.
6. In addition to standard reporting mechanism for this type of event, submit information to the Cancer Center IND Coordinator and Cancer Center PDC. These persons will facilitate FDA and IRB reporting, respectively, as warranted by the event. If Adverse Event Expedited Report – Single Agent or Multiple Agents report form was completed, this form does not need to be completed.

10.3 Adverse events to be graded at each evaluation and pretreatment symptoms/conditions to be evaluated at baseline per Common Terminology Criteria for Adverse Events (CTCAE) v3.0 grading unless otherwise stated in the table below:

CTCAE Category	Adverse event/Symptoms	Baseline	Each evaluation
Constitutional Symptoms	Fatigue	X	X
Dermatology/Skin	Injection site reaction		X
	Rash/desquamation	X	X
Pain – Select Musculoskeletal	Bone	X	X
	Joint	X	X
	Muscle	X	X

10.31 Submit via appropriate MCCC Case Report Forms (i.e., paper or electronic, as applicable) the following AEs experienced by a patient and not specified in Section 10.3:

10.311 Grade 2 AEs deemed *possibly, probably, or definitely* related to the study treatment or procedure.

10.312 Grade 3, 4, and 5 AEs and deaths within 30 days of the patient's last treatment, regardless of attribution to the study treatment or procedure, with the exception of signs or symptoms of definitely related to the patient's disease or disease progression.

10.313 Any death more than 30 days after the patient's last study treatment or procedure which is felt to be at least possibly treatment related must also be submitted as a Grade 5 AE, with a CTCAE type and attribution assigned.

10.32 Refer to the instructions in the electronic data entry screens regarding the submission of late occurring AEs following completion of the Active Monitoring Phase (i.e., compliance with Test Schedule in Section 4.0).

Information included at the request of the Department of Defense, a financial sponsor of the study

Reporting of serious or unexpected adverse events and unanticipated problems.

a. Serious or unexpected adverse events and unanticipated problems can occur in any and all types of studies, not just experimental interventions or clinical trials.

b. Include a definition of what constitutes an adverse event in the study.

(1) For IND or IDE research include definitions as described in 21 CFR 312.32.

(2) All IND protocols must address the following requirements.

"An adverse event temporally related to participation in the study should be documented whether or not considered to be related to the test article. This definition includes

intercurrent illnesses and injuries and exacerbations of preexisting conditions. Include the following in all IND safety reports: Subject identification number and initials; associate investigator's name and name of MTF; subject's date of birth, gender, and ethnicity; test article and dates of administration; signs/symptoms and severity; date of onset; date of resolution or death; relationship to the study drug; action taken; concomitant medication(s) including dose, route, and duration of treatment, and date of last dose."

c. Describe agencies or offices to be notified with point of contact information in the event of a serious and unexpected adverse event.

All protocols should contain the following language regarding the HSRRB reporting requirements for adverse events and unanticipated problems. (Note that unanticipated problems can occur in a study that does not require a research/clinical intervention.)

"Unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all volunteer deaths should be promptly reported by phone (301-619-2165), by email (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board. A complete written report should follow the initial telephone call. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21702-5012"

Refer to the "HSRRB Information Sheet for Investigators: Unanticipated Problems" for examples of unanticipated problems located on our website.

"The medical monitor for this project, Dr. Ravi Rao, is required to review all unanticipated problems involving risk to volunteers or others, serious adverse events and all volunteer deaths associated with the protocol and provide an unbiased written report of the event. At a minimum the medical monitor should comment on the outcomes of the event or problem and in the case of a serious adverse event or death comment on the relationship to participation in the study. The medical monitor should also indicate whether he/she concurs with the details of the report provided by the study investigator. Reports for events determined by either the investigator or medical monitor to be possibly or definitely related to participation and reports of events resulting in death should be promptly forwarded to the HSRRB."

The medical monitor will forward reports to the U.S. Army Medical Research and Material Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

11.0 Treatment Evaluation

11.1 For the purposes of this study, patients should be re-evaluated every 4 weeks during immunizations (treatment) and every 12 weeks during follow-up.

11.2 At the time of reevaluation, patients will be classified in the following manner:

11.21 No evidence of disease (NED).

11.22 Breast cancer recurrence (REC). Local/regional breast cancer recurrence is defined as the development of tumor (except LCIS) in the ipsilateral breast (after lumpectomy); in the soft tissue/chest wall and/or skin of the ipsilateral chest wall; or tumor in the

ipsilateral internal mammary, infraclavicular, or axillary nodes or soft tissue of ipsilateral axilla. Suspected tumor recurrence in the ipsilateral breast, chest wall structures or lower (level I ± II) axillary nodal areas must be confirmed by biopsy or cytology. Histologic or cytologic confirmation of tumor is recommended for internal mammary or infraclavicular/high axillary nodal recurrence. A distant recurrence is defined as development of tumor in areas other than the local/regional area that is documented by a positive cytology aspirate, biopsy, or imaging studies.

11.23 New primary (NEWP): A new primary is defined as the development of contralateral breast cancer or a second cancer other than squamous or basal cell carcinoma of the skin, carcinoma in situ of the cervix or LCIS of the breast that is histologically confirmed.

11.3 Further treatment after the documentation of a breast cancer recurrence or second primary cancer is left to the discretion of the treating physician.

12.0 Descriptive Factors: None.

13.0 Treatment/Follow–up Decision at Evaluation of Patient

13.1 Patients who have not recurred at time of their reassessment and have not experienced intolerable toxicity may continue protocol treatment at the same dose level for a maximum of 6 cycles or until progression of disease, a second primary or an intolerable adverse event occurs.

13.2 Patients who develop progression of disease, a second primary or intolerable toxicity will be removed from protocol treatment and go to the event monitoring phase of the study. Subsequent treatment is at the discretion of the treating physician.

13.3 Patients may refuse further protocol treatment at any time and go to the event-monitoring phase of the study.

13.4 If a patient is declared ineligible by the study team, on-study material, treatment evaluation forms, and end of treatment form should be submitted. No further follow-up after notification of ineligibility is required.

13.5 If a patient is declared a cancel by the study team, on-study material should be submitted. No further follow-up is required.

13.6 If patient is found on central review of MUC1 negative, the patient will be considered a cancel.

13.7 There will be no replacement of patients who discontinue or are removed from the protocol for any reason.

14.0 Correlative/Translational Studies

14.1 Description of Assays

Active vaccines for the immunotherapy of solid tumors have met with only limited success. It is our hypothesis that the causes of this failure are multifactorial and can be improved by the inclusion of stringent patient selection criteria, careful dose titration based on immunologic response monitoring, and correlation of immunologically based dosing parameters with clinical outcome. The following sections define the strategies that will be employed in this trial to evaluate immunologic response to MUC1, and HER-2 peptides.

14.11 Immune Responses to T Helper and CTL Epitopes

14.111 Elispot

Estimates of frequencies of peptide-specific, IFNγ- and IL-5-producing cytotoxic T lymphocytes and helper T lymphocytes will be obtained by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells [74, 75]. IL-5 production, rather than that of IL-4, will be assayed because of the increased signal:noise ratio [74]. $CD8^+$ and $CD4^+$ T cells will be positively selected by magnetic activated cell sorting (MACS, Miltenyi Biotech) from cryopreserved and thawed peripheral blood lymphocyte buffy coat. Antigen-presenting cells (APCs) will also be isolated from $CD4^-/CD8^-$ cell population by MACS (beads and reagents purchased from Miltenyi Biotech). $CD8^+$ and $CD4^+$ responder T cells will be stimulated with irradiated APCs pulsed with the target peptides used for vaccination. After 5 days of co-culture, the responding cells will be diluted, titrated, and re-stimulated with APCs pulsed with target peptides for 24 hours in 96 well microtiter ELISPOT plates coated with IFNγ- or IL-5-specific capture antibody (ELISPOT Kit purchased from MABTECH, Stockholm, Sweden). The target peptides for re-stimulation include the peptide used for primary stimulation (MUC1 and HER-2 peptides) and a negative control peptide (YIGEVLVSV). The wells are washed and treated with ALP-conjugated secondary antibody and cytokine-producing spots detected using appropriate substrate (all reagents are provided in the kit). After stopping the reaction, the developed microtiter plates are shipped to Zellnet Consulting in New York for evaluation of number of spot-producing cells for each responder cell titration. All analyses are performed by the consulting firm and data provided electronically to the investigator. The difference between the frequency of spot-producing cells obtained with the target peptides and control peptide will determine the frequency of peptide-specific, cytokine-producing $CD4^+$ or $CD8^+$ T cells.

14.112 Tetramers

The estimation of frequencies of CTLs that recognize specific peptides bound to class I molecules became increasingly easier and more quantifiable with the construction and application of class I tetramers [11, 76, 77]. Class I MHC tetramers are composed of a complex of four HLA MHC class I molecules each bound to the specific peptide and conjugated with a fluorescent protein (MHC Tetramer-Streptavidin-Phycoerythrin (SA-PE)). We will use MUC1 M1.1 peptide (STAPPVHNV) and HER-2/neu peptide 9_{435} (ILHNGAYSL). To detect epitope spreading, we will also use HER-2/neu $peptide_{369-377}$ (KIFGSLAFL). As a negative control, we will use the multi-allele negative tetramer from Beckman Coulter (T01044). For positive control we will use the HLA-A0201 CMV PP65 tetramer (NLVPMVATV) from Beckman Coulter (T01009). Tetramers of HLA-A2 molecules are commercially available (Beckman Coulter). On the day of staining, test PBLs are thawed, washed, and resuspended in the manufacturer's recommended staining buffer (PBS) at 1×10^6 cells/ml. Tetramers and any additional antibodies (such as anti-CD8 or anti-CD3 conjugated to a different fluor such as FITC) are added to the cell volumes and incubated for 30 min at room temperature. The cell suspension is then washed with PBS and resuspended in PBS with 0.5% formaldehyde (Fixative Reagent) and analyzed by flow cytometry with FACSCAN

instrumentation and CellQuest software (BD Biosciences); a minimum of 5 x 10^5 cells/sample are analyzed for accurate estimation of $CD8^+$ CTLs with low frequencies. The analysis involves (1) gating on lymphocytes using forward and side-scatter; (2) gating on FITC-positive PBLs that stain with anti-CD3 or anti-CD8, and (3) analyzing the gated cells for PE and FITC staining. The frequency of doubly stained cells ($tetramer^+/CD8^+$) will be estimated for each of three replicate tubes for calculation of the mean frequency ($\pm$sd).

14.12 Antigenic Profiling

14.121 Expression of Class I HLA Antigens on tumor tissue.

Initial entry criteria require HLA-A typing of peripheral blood with subsequent confirmation of HLA class I antigen expression on tumor cells by immunohistochemistry. One of the mechanisms by which tumors are postulated to evade the immune response is by down regulation of classical HLA molecules necessary for antigen presentation.

14.122 Tumor Expression of MUC1 and HER-2

Tumor blocks will be used to determine the levels of expression of HER-2 and MUC1 on breast cancer tumor cells obtained at the time of most recent surgical resection. HER-2 expression will be determined using a clinical grade test +1 to +3 and levels of expression will be graded on a semi-quantitative scale. MUC1 expression will be determined by positive staining with one of several antibodies to MUC1 (HMFG-2, BC-2, or B27.29). Negative controls will be incubated with PBS instead of monoclonal antibody. Staining of cytoplasm and plasma membrane will be evaluated. Cells will be considered positive when at least one of these components is stained. Antibody staining patterns will be scored in a semi quantitative manner from +1 to +3.

14.13 Sample Schedule

14.131 Blood

100 mL of blood (about 7 tablespoons) will be collected (heparin) prior to registration and prior to each subsequent immunization as well as every 3 months after conclusion of active therapy until 24 months following registration. Prior to each study blood collection a complete blood count will be performed. If the serum hemoglobin is less than 10.0, the study sample will not be collected. Study sample collection will be postponed for the next study visit.

14.132 Tumor

Tumor blocks will be collected from the patient's most recent surgery prior to study registration. Sections from the tumor blocks will be stained for MUC1. Any/all remaining tissue samples will be returned to the clinical file. Any/all excess samples will be destroyed.

14.14 Sample Preparation

14.141 Blood

Peripheral blood lymphocytes (PBLs) are enriched by flotation over Ficoll-Hypaque and frozen in aliquots in 10% DMSO for storage at -150°C. Percentages of $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, and dendritic cells are estimated by flow cytometry with a panel of specific monoclonal antibodies. In addition, proliferation assays (3H-thymidine uptake) are performed to estimate T cell responses to polyclonal stimulus (phytohemagglutinin), target antigens (MUC1 and HER-2/neu) and a recall antigen (tetanus toxoid). These two sets of experiments are important for estimating the representation of individual lymphoid populations and evaluating overall T cell responsiveness. $CD8^+$ (CTLs) and $CD4^+$ (HTLs) are positively purified from cryopreserved and thawed PBLs by magnetic bead separation (Miltenyi Biotek). Additionally, serum will be collected and stored from each of these samples. Cells will then be frozen and stored at -150° for future use.

14.3 Delayed-type hypersensitivity (DTH) skin testing

Skin testing (baseline - prior to registration) will be coordinated through the Mayo Immunization/Allergy Clinic (L-15). A typical panel includes candida, mumps, PPD, and trichophyton. Other antigens may be substituted in the event of antigen unavailability. Patients will return for 1-2 follow-up measurements consistent with L-15 procedures. Patients must have a "positive" reaction to at least one of the antigens tested, to be considered eligible for participation. Patients with only "doubtful" or "negative" reactions will not be considered eligible.

15.0 Drug Information

15.1 MUC-1 (STAPPVHNV) - *Investigational supply*

15.11 Other Names: epithelial membrane antigen (EMA), polymorphic epithelial antigen (PEM), DF3 antigen, Ca1, MAM-6, H23, episialin

15.12 Formulation and Storage: Samples will be vialed (glass vials with Teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at -20°C until use.

15.13 Drug Procurement and Accountability: purchased from Clinalfa

15.2 HER-2 Peptide-1 (ILHNGAYSL) - *Investigational supply*

15.21 Other Names: erbB2, neu

15.22 Formulation and Storage: Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at –20°C until use.

15.23 Drug Procurement and Accountability: purchased from Clinalfa

15.3 HER-2 Peptide-2 (KVPIKWMALESILRRRF) - *Investigational supply*

15.31 Other Names: erbB2, neu

15.32 Formulation and Storage: to be determined
Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at –20°C until use.

15.33 Drug Procurement and Accountability: purchased from Clinalfa

15.4 Montanide ISA-51 Adjuvant [MONTAN] - *Investigational supply*

15.41 Formulation and Storage

Montanide ISA-51 is an oil-based adjuvant product similar to Incomplete Freund's Adjuvant. Which when mixed with a water-based solution on 1:1 w/w ration, forms a water-in-oil emulsion. It consists of highly purified oil, Drakol VR, and a surfactant, mannide oleate. Montanide ISA-51 is manufactured by Seppic, Inc., and is provided in amber glass ampoules containing 3 mL of the solution. Montanide ISA-51 will be purchased from Seppic Inc.

15.42 Mode of Action

Acts to enhance immune response to vaccination; the precise mode of action is unknown.

15.43 Storage and Stability

The solution is stored at controlled room temperature. Exposure to cold temperatures may result in a clouded solution, which should be discarded. An expiration date is printed on the ampoule label.

15.44 Compatibilities/Incompatibilities

The oil may break down the rubber tip of the plunger on syringes; it is advisable to use a different syringe for each ampoule. Do not allow the Montanide ISA-51 to be in direct contact with the rubber tip of the plunger for more time than is necessary to withdraw the solution and inject it into the peptide vial. Fresh syringes will be needed to withdraw the emulsified vaccine from the vaccine vial. Once the emulsion is made, there is less interaction of the oil directly with the rubber tip of the plunger.

15.45 Drug Procurement and Accountability

Montanide ISA-51 will be purchased from Seppic Inc. The Cancer Center Pharmacy Shared Resource will store the drug and maintain records of inventory and disposition of all agent received.

15.5 GM-CSF (sargramostim, Leukine®)

15.51 Preparation and Storage

Liquid (used in this study) is available in vials containing 500 mcg/mL (2.8×10^6 IU/mL) sargramostim. Carton of 5 multiple-dose vials; each vial contains 1 mL of preserved 500 mcg/mL LEUKINE Liquid (NDC 50419-050-30). LEUKINE should be refrigerated at 2-8°C (36-46°F). Do not freeze or shake. Do not use beyond the expiration date printed on the vial.

15.52 Known Potential Toxicities

Fever, chills, asthenia, malaise, rash, peripheral edema, dyspnea, headache, pericardial effusion, bone pain, arthralgia, and myalgia.

15.53 Drug Procurement:

Leukine 500 mcg vials are available commercially. Drug will be purchased for this project using study grant funds. Patients will not be charged for the GM-CSF.

15.6 CpG-7909 (Promune™)

15.61 Preparation and Storage: CpG-7909 is formulated as a sterile phosphate buffered saline solution (5mg/mL) stabile for parenteral administration. The sterile and pyrogen free solution contains no preservatives. Vials are intended for single entry: penetration of the vial's stopper should only be done once to maintain sterility. The drug product is packaged in clear, Type I USP glass vials with teflon-coated stopper closure and flip-caps. The drug product should be stored under refrigeration (2°-8°C). CpG-7909 is stable for at least one year if stored frozen.

15.62 Known potential toxicities: The list of reported serious adverse events with the use of CpG-7909 demonstrates the following toxicities:

15.621 **Related**: reactive follicular lymphatic hyperplasia.

15.622 **Possibly Related**: anemia, superior vena cava syndrome, dyspnea, malignant ascites, post-operative bleeding, hepatic failure, renal failure, post-operative wound infection, GI hemorrhage, prolonged coagulation time, bacteriemia, ureteric obstruction, congestive heart failure, DVT, vomiting, dehydration, vein compression, hydronephrosis, urinary retention, proctalgia, hypercalcemia, pleural effusion, subacute inflammatory demyelinating polyneuropathy, pelvic inflammatory disease, unstable angina, myocardial infarction, atrial fibrillation and grand mal seizures.

15.63 Drug Procurement: to be purchased from Coley Pharmaceutical Group Inc.

15.7 Vaccine Preparation Instructions

15.71 General Vaccine Preparation Information

Emulsify the peptide(s)/GM-CSF or CpG mixture with Montanide ISA-51. Prepare the vials as directed for each group below. Place the vial upside down in a tube platform holder of a vortex machine and vortex at highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Because neither the peptide solution nor the Montanide ISA-51 contains preservatives or bacteriostatics, the prepared peptide vaccines should be administered as soon as possible.

15.711 Arm A

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid GM-CSF (500 mcg/mL) from the refrigerator and allow to reach room temperature. Withdraw 700µL of GM-CSF and add to

the peptide vial containing 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also, 1.0 mL of Montanide ISA-51 to the peptide vial. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two tuberculin syringes with equal volumes of this emulsion prior to use. Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused GM-CSF and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

Arm B

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid CpG-7909 (5mg/vial/mL) from the refrigerator and allow to reach room temperature. Withdraw 0.5mL of CpG-7909 and add to the first peptide vial containing 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also add 1.0 mL of Montanide ISA-51. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two tuberculin syringes with equal volumes of this emulsion prior to use. Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused CpG and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

Arm C

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid GM-CSF (500 mcg/mL) from the refrigerator and allow it to reach room temperature. Remove a vial of liquid CpG-7909 (5mg/vial/mL) from the refrigerator and allow it to reach room temperature. Withdraw 0.5mL of CpG-7909 and add to the first peptide vial containing 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also add 1.0 mL of Montanide ISA-51 to the peptide vial. Finally, add 0.6mL of GM-CSF to the mixture. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two to three tuberculin syringes with equal volumes of this emulsion prior to use. Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after

2 minutes. Discard unused GM-CSF, CpG-7909 and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

15.8 Vaccine Administration Information

15.81 Dose Specifics

Each peptide vaccine will consist of a total volume of approximately 2 to 4 mL, containing the correct dose of the peptide(s), Montanide ISA-51, and GM-CSF or CpG. Be sure to confirm the proper cohort and dose level before preparing the product.

15.82 Administration

Vaccinations will be given subcutaneously on day 1 of each treatment cycle. Due to the large volume, each peptide vaccine is administered in 2 to 3 shots in a contiguous location. The peptide vaccine should be injected in the vicinity of one of the major nodal basins. This basin must not have been dissected.

15.9 Vaccine Side Effects:

15.91 Because of the low dose of GM-CSF used and the slow release nature of the vaccine emulsion, side effects normally seen with systemic treatment doses of GM-CSF should not play a factor in this vaccination treatment. Expected side effects are related to the peptides and Montanide ISA-51. It is possible that the GM-CSF and CpG-7909 may potentiate the reaction seen at the injection site.

15.92 Dermatology/Skin: Injection site reaction, rare granuloma formation, possible development or worsening of pre-existing vitiligo, rash.

15.93 Hepatic: transient rises in liver transaminases.

15.94 Constitutional: Low-grade fever.

16.0 Statistical Considerations and Methodology

16.1 Study design: This is a pilot study designed to determine the immunologic effects of a MUC1/HER-2/neu peptide based tumor vaccine in the presence of the immune adjuvant GM-CSF suspended in Montanide ISA-51, CpG suspended in Montanide ISA-51 or the combination of GM-CSF and CpG co-emulsified in Montanide ISA 51.

16.11 A secondary goal of the study is to determine the toxicity profile of each treatment combination

16.2 The study design chosen for this proposal is a stratified randomized design. Toxicities will be carefully monitored and accrual will be suspended if 2 or more of the first six patients experience a grade 4 hematologic toxicity lasting for 5 or more days. In the event of at least two patients experiencing immunologic toxicity $\geq$ grade 2 or any toxicity $\geq$ grade 3 accrual will be temporarily suspended for the given treatment arm.

16.3 Accrual: Fifteen patients with MUC1/HER-2 positive breast cancer with no evidence of disease will be randomized to each one of the 3 treatment schedules. The total number of eligible patients to be accrued will be 45. Patients will be allocated to each treatment schedule using a dynamic allocation procedure that balances the marginal distribution of type of dominant disease between treatment combinations. The expected accrual rate for this study is about 15-20 patients at Mayo Clinic Rochester and about 5-7 patients each at Mayo Clinic Scottsdale and Mayo Clinic Jacksonville per year. The study is expected to begin enrollment during 2005, and will accrue patients for approximately 2 years.

16.4 Study Endpoints:

16.41 Primary Endpoints

16.411 The immunologic parameters of interest are: (1) the percentage of $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, and dendritic cells in a patient's peripheral blood sample as estimated by flow cytometry with a panel of monoclonal antibodies and (2) the frequency of both peptide-specific IFN-gamma producing T cells and peptide-specific IL-5 producing T cells estimated by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells for the MUC1 and HER-2 peptides.

16.412 The number and severity of hematologic and non-hematologic toxicities reported using the NCI-CTC version 3.0 criteria

16.42 Secondary Endpoints

16.421 Disease-free survival is defined as the time from registration to the documentation of a first failure where a failure is the recurrence (REC) of breast cancer or a diagnosis of a second primary cancer (NEWP).

16.422 Overall survival is defined as the time from registration to death due to any cause.

16.43 Immunologic Parameters

16.431 All eligible patients who have completed one cycle of treatment are evaluable for the analysis of the immunologic parameters.

16.432 For each of the immunologic parameters, a plot of the parameter level against time will be constructed such that each patient is represented by a line connecting that patient's data points. These plots will enable visual assessment of patterns of change and variability within a parameter as well as a visual assessment of whether the immunologic parameters peak or fall at similar time points.

16.433 Also, for each of the immunologic parameters, a plot of the percent change from pre-treatment levels against time will be constructed such that each patient is represented by a line connecting that patient's data points. These plots will enable visual assessment of time trends within a parameter controlling for pretreatment levels.

16.44 Adverse Events

16.441 All eligible patients who received at least one vaccination are evaluable for toxicity.

16.442 The frequency of those hematologic and non-hematologic toxicities considered at least possibly related to treatment will be tabulated by severity.

16.443 The circumstances surrounding any treatment-related death will be reported.

16.444 As this is a pilot study, no formal hypothesis tests comparing treatment schedules are planned. An immunization strategy will be considered for further testing if at least 70% patients treated with that strategy had a ≥2-fold increase in the percentage of vaccine-peptide specific $CD8^+$ T cells during the course of treatment, with tolerable toxicity.

16.445 The principal investigator and study statistician will review the study every 3 months to identify potential accrual, toxicity, or endpoint problems. In addition, this study will be monitored by the Cancer Center Data Safety Monitoring Board. All patient related clinical data will be entered and maintained online, with reports generated as needed to comply with reporting guidelines.

16.446 If the protocol requires any modifications, deviations or termination prior to completion, all administrative activities will comply with the Protocol Review and Monitoring System of the Mayo Clinic Comprehensive Cancer Center. In addition, all local IRB communications, including deviations from protocol, will be forwarded to the Department of Defense HSRRB, upon local approval.

16.45 Inclusion of Minorities

This study will be available to all eligible patients, regardless of race or ethnic group. There is no information currently available regarding differential agent effects in subjects defined by gender, race, or ethnicity. The planned analyses will, as always, look for differences in treatment effect based on racial groupings. The sample sizes of this pilot study, however, are not sufficient to provide power for such subset analyses.

To predict the characteristics of patients likely to enroll in this trial we have reviewed registration to (non-North American Breast Cancer Intergroup) NCCTG breast cancer clinical trials by race. This revealed that roughly 3% of patients registered into cancer trials during the past five years could be classified as minorities, which would suggest that only 1 or 2 patients in the study sample are expected to be classified as minorities. This small sample precludes the possibility of a separate subset analysis beyond simple inspection of results for the 1 or 2 minority patients.

17.0 Pathology Considerations for Quality Control

17.1 There will be a central review of tumor tissues stained for MUC1 and HER-2/neu

17.11 Describe materials to be submitted.

Pathology Reporting Form
Surgical Pathology and Operative Report
All diagnostic slides

Slides should be placed in appropriate slide container and labeled with the protocol number, study patient number, and patient initials.

18.0 Records and Data Entry Procedures

18.1 Data Entry Timetable

Forms	Active-Monitoring Phase (Compliance with Test Schedule)			Event-Monitoring Phase[1] (Completion of Active-Monitoring Phase)				At Each Occurrence			
	Initial Material	Follow-up material									
	≤2 weeks after registration	At each evaluation	At end of treatment	q.3 months until PROG	At PROG	After PROG q. 3 mos.	Death	ADR/ AER	New Primary	Grade 4 or 5 Non-AER Reportable Events/ Hospitalization	Late Adverse Event
On-Study Form	X										
Specimen Submission Form [2]	X										
Baseline Adverse Events/ Symptoms	X										
Measurement Form	X	X	X								
Evaluation/Treatment Form		X	X								
DTH Laboratory Form[3]	X										
Interval Laboratory Form[4]	X										
Nadir/Adverse Event Form		X	X								
End of Active Treatment Form			X								
Event-Monitoring Form			X	X	X	X	X		X		X
Concurrent Treatment Log	X	X	X								
ADR/AER (See Section 10.0)								X			
Secondary AML/MDS Report Form (See Section 10.0)								X			
Grade 4 or 5 Non-AER Reportable Events/ Hospitalization Form (See Section 10.0)										X	

1. If a patient is still alive after 2 years after registration, no further follow-up is required.
2. Research blood samples will be performed before registration as well as prior to **cycles 3, 5 and 6** of therapy as well as every other cycle of long-term follow-up **starting at 3 months** after completion of Rx up to two years.
3. At baseline and prior to cycle 6 only.
4. At baseline, prior to each subsequent treatment and at 4 weeks after last treatment.

19.0 Budget Considerations

19.1 Costs charged to patient: routine clinical care.

19.2 Tests and procedures to be research funded: HLA typing, tumor typing. DTH testing and serum pregnancy tests. Funding will be provided by the Department of Defense (DOD).

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Appendix I

ECOG PERFORMANCE STATUS

Grade	
0	Fully active, able to carry on all pre-disease activities without restriction (Karnofsky 90-100).
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work (Karnofsky 70-80).
2	Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50 percent of waking hours (Karnofsky 50-60).
3	Capable of only limited self-care, confined to bed or chair 50 percent or more of waking hours (Karnofsky 30-40).
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair (Karnofsky 10-20).
5	Dead

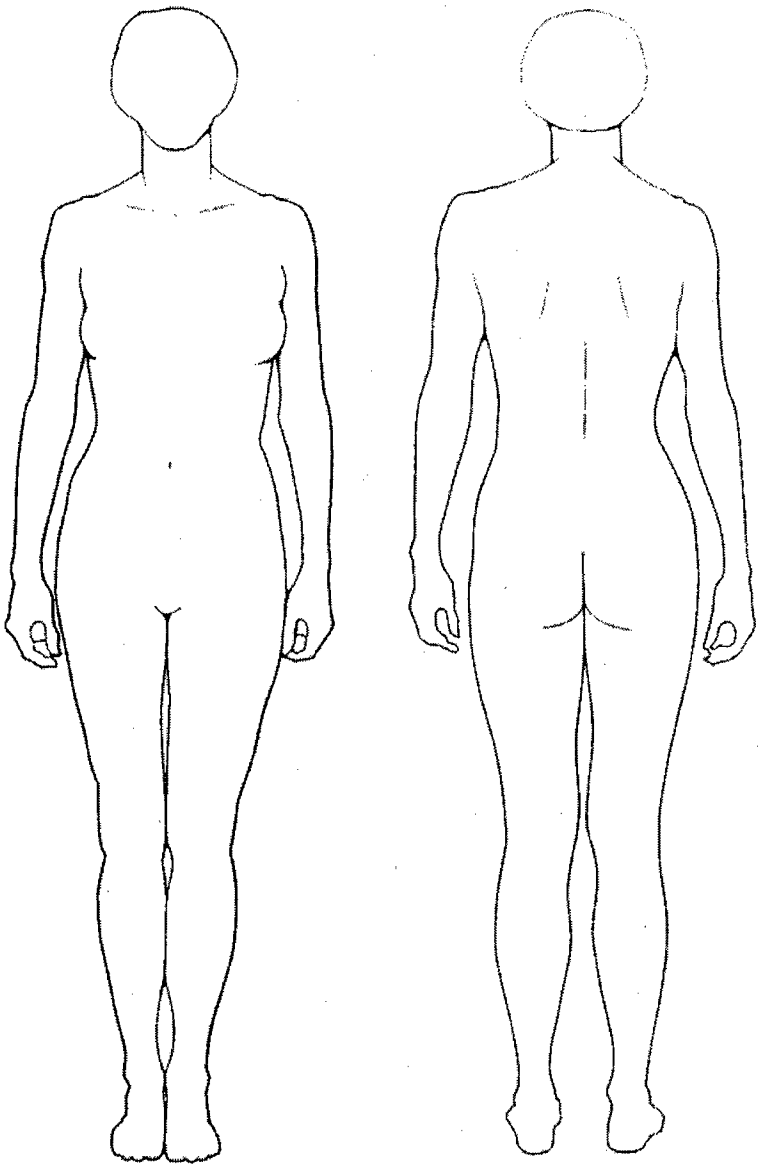
Appendix II

Site Injection Record

Protocol #: _____ Patient #: _____ Patient Initials: ___ ___ ___
F M L

Please indicate on the diagrams below all sites of vaccine injection (circle).

Date of determination: ___/___/___
M D Y



Anterior Posterior

MAYO CLINIC
Rochester, Minnesota

Retain In Correspondence
Section of Medical Record

Name and Registration No.

Consent Form for Participation in a Research Study

TITLE: MC0338, "MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas"

IRB #: 782-05 00

RESEARCHER: Dr. S. N. Markovic and colleagues.

PROTOCOL LAST APPROVED BY INSTITUTIONAL REVIEW BOARD: April 22, 2005

THIS FORM APPROVED: April 22, 2005

This is an important form. Please read it carefully. It tells you what you need to know about this research study. If you agree to take part in this study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

We are inviting you to participate in this study because of your history of resected breast cancer for which you have already received standard therapy and have no evidence of relapse. Your good overall health and no other history of cancer make it possible for us to offer you participation on this clinical study.

This study is being done to evaluate three different preparations of a breast cancer vaccine to stimulate anti-cancer (T-cell) immune responses and any side effects associated with these vaccinations. The breast cancer vaccine (MUC-1/HER-2/neu peptides) will be combined with one of two immune boosting agents (CpG or GM-CSF) or with immune boosting agents together. These immune boosting agents are believed to be able to make the vaccine more effective.

The breast cancer vaccine and one of the immune boosting agents (CpG) have not been approved by the Food and Drug Administration (FDA) for commercial use; however, FDA has permitted their use in this research study. GM-CSF is commercially available for use in clinical practice.

This study is sponsored by the Department of Defense.

How many people will take part in this research study?

The plan is to have up to 45 people take part in this study at Mayo Clinic Rochester, Jacksonville, and Arizona. Up to 60 people may be screened to find enough eligible people to begin the study.

What will happen in this research study?

Before you enter the study, you will have a physical examination and blood tests to make sure that you qualify to take part in this study. About 6 tablespoons of blood will be taken for testing.

If you qualify to take part, you will have blood taken for immunologic testing (to see how your immune system is working) for the study as well as a skin test (allergy test). You will then be randomly assigned (as in the toss of the dice) to be treated with one of three breast cancer vaccines combinations.

Each vaccination will consist of one or two subcutaneous (under the skin) injections of the cancer vaccine and boosting agent combination. The vaccine will be given with Montanide ISA 51, which is an oil that is mixed with the vaccine so that the vaccine can be released into the body slowly. The vaccine will be injected under the skin in areas where there has been no surgery. Usual areas of vaccination include the skin of the upper arms and legs. Vaccinations will be repeated every 4 weeks for 6 months. Before every vaccination, you will be seen by your doctor, examined and about 6 tablespoons of blood will be collected to study the development of the anti-cancer immune response. Skin tests will be done prior to vaccinations #6 (see table below). Evaluations for the cancer (body scans) will be done if and when your doctor thinks it is necessary.

Pre-Study	• Routine[1] and research blood tests • Cancer evaluation (scans) • Physical examination • Skin test (Mayo Clinic Rochester, only)
Vaccination #1 (month 1)	• Vaccine treatment
Vaccination #2 (month 2)	• Routine blood test collection • Physical examination • Vaccine treatment
Vaccination #3 (month 3)	• Routine and research blood test collection • Physical examination • Cancer evaluation (scans) • Vaccine treatment
Vaccination #4 (month 4)	• Routine blood test collection • Physical examination • Vaccine treatment

Vaccination #5 (month 5)	• Routine and research blood test collection • Physical examination • Vaccine treatment
Vaccination #6 (month 6)	• Routine and research blood test collection • Physical examination • Cancer evaluation (scans) • Vaccine treatment • Skin test (Mayo Clinic Rochester, only)
Every 3 months after the first six months until 5 years after first vaccination	• Physical examination • Routine blood tests • Cancer evaluation (scans)
Every 3 months after the first six months until 2 years after first vaccination	• Research bloods

1. Routine blood tests include: complete blood count and blood chemistries.

How long will I be in this research study?

You will be treated on the study for 6 months, and you will be seen in follow-up until 5 years after your first vaccination.

Are there reasons I might leave this research study early?

Taking part in this research study is your decision. You may decide to stop at any time. You should tell the study doctor if you decide to stop and you will be advised whether any additional tests may need to be done for your safety.

In addition, the investigators or Mayo may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped.

Will any biological sample(s) be stored and used for research in the future?

No. Your samples will be used as described for this study, and then will be destroyed.

What are the risks of this research study?

While you are taking part in this study, you are at risk for the following side effects. You should talk to your study doctor and/or your medical doctor about these side effects. There also may be other side effects that are not known. Other drugs may be given to lessen side effects. Many side effects go away shortly after the vaccine treatments are stopped, but in some cases side effects can be serious, long lasting, or may never go away. The side effects can be mild or can lead to death.

You will be closely watched by the study team for any side effects. If side effects happen, the study team will take the necessary steps to treat them. This may include stopping the medication and/or stopping the study.

If any new side effects are found as the study continues, you will be notified.

The same possible side effects of the following: MUC-1/HER-2/neu, breast cancer vaccine, Montanide ISA 51 (the oil), CpG (immune booster), and GM-CSF (immune booster) include skin rash, itching, sweating, headache, muscle aches, joint aches, stomach pain, fatigue, cough, injection site reactions (pain, rash, redness, firmness, warmth, bleeding, ulceration, tenderness to touch, numbness, tingling, itching).

However, when GM-CSF has been given at higher doses as a daily injection the following side effects have also been reported: diarrhea, general weakness, fever, chills, nausea, vomiting, loss of appetite, headache, pain in the bones, joints and muscles. Most of the symptoms were mild or moderate in severity and were less after taking acetaminophen (Tylenol). Other side effects which happened very rarely were: difficulty breathing, rapid or irregular heart beat or other heart problems, swelling. Even less common, reported side effects have been the following: 1) increased white cells in the lungs with breathing problems; 2) a syndrome of shortness of breath, low oxygen in the blood, redness in the skin, low blood pressure and dizziness when you stand up or a loss of balance and partial loss of consciousness; 3) serious allergic reactions (like a very severe asthma attack); 4) blood clotting; 5) facial flushing; 6) kidney or liver problems; 7) worsening of fluid accumulation in the arms, legs, lungs or around the heart which may cause problems with breathing or heart failure; 8) patients with heart, lung, kidney or liver problems may have worsening of their symptoms following GM-CSF; and 9) nerve toxicity (weakness, shooting pains, numbness, increased sensitivity to touch, loss of balance, dizziness)

Skin testing: The risks and discomfort of skin testing are minimal and usually limited to bleeding, bruising, or infection at the injection site.

Blood draws: A blood drawing may cause slight pain and a small risk of bleeding, bruising, or infection at the injection site.

There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child of a woman who takes part in this study. Women who can become pregnant must use one of these birth control plans during this study: diaphragm, birth control pills, injections, intrauterine device (IUD), surgical sterilization, under the skin implants, abstinence. Another choice is for your sexual partner to use appropriate forms of contraception. Breast-feeding mothers must stop breast-feeding to take part in this study. Women who can become pregnant must have a pregnancy test before taking part in this study. For the pregnancy test, blood will be taken from a vein in your arm

with a needle within 7 days before you enter the study. You will be told the results of the pregnancy test. If the pregnancy test is positive, you will not be able to take part in the study.

This study may involve risks to you (or to an embryo or fetus if you become pregnant) that are currently unforeseeable.

There may be other risks with the combination of drugs/vaccine which we are not yet aware of.

Are there benefits to taking part in this research study?

This study may not make your health better. However, the information learned may benefit future patients with breast cancer.

What other choices do I have if I don't take part in this research study?

You do not have to be in this study to receive treatment for your condition. Your other choices may include participation on other clinical studies or no treatment at all, but you will have regular appointments with your doctor who will check your condition. You should talk to your doctor about your choices before you decide if you will take part in this study.

Will I need to pay for the tests and procedures?

You will not need to pay for any tests and exams that are done just for this research study, including the research blood tests, skin tests and office visits done only for this research study. However, you and/or your health plan will need to pay for all other tests and procedures that are part of this study because they are needed for your regular medical care. You or your health plan might have to pay for other drugs or treatment given to help you control side effects. You will not also need to pay for the vaccine used in this study. Before you take part in this study, you should call your health insurer to find out if the cost of these tests, procedures, and/or the device will be paid for by the plan. Some health insurers will not pay for these costs. You will have to pay for any costs not covered by your health insurer. If you have questions while at the Clinic, please go to the Admissions and Business Services office, or you may call Patient Account Services at (507) 287-1819.

What happens if I am injured because I took part in this research study?

If you have side effects from the study vaccine treatments, you need to report them to the researcher and your regular physician, and you will be treated as needed. Mayo will bill you and your insurer for these services at the usual charge. Mayo will not offer free medical care or payment for any bad side effects from taking part in this study. You should discuss this issue thoroughly with the study doctor before you enroll in this study.

If you are hurt or get sick because of this research study, you can receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic. If you have questions about this medical care, talk to the principal investigator for this study, Svetomir N. Markovic, M.D., Ph.D. If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

This does not mean that you are giving up any legal rights that you may have.

What are my rights if I take part in this research study?

Taking part in this research study does not take away any other rights or benefits you might have if you did not take part in the study. Taking part in this study does not give you any special privileges. You will not be penalized in any way if you decide not to take part or if you stop after you start the study. Specifically, you do not have to be in this study to receive or continue to receive medical care from Mayo Clinic. If you stop the study you would still receive medical care for your condition although you might not be able to get the study drug.

You will be told of important new findings or any changes in the study or procedures that may affect you or your willingness to continue in the study.

Who can answer my questions?

You may talk to Dr. Svetomir N. Markovic at any time about any question you have on this study. You may contact Dr. Markovic (or an associate) by calling the Mayo operator at telephone (507) 284-2511.

You can get more information about Mayo policies, the conduct of this study, or the rights of research participants from Marcia Andresen-Reid, the administrator of the Mayo Foundation Office for Human Research Protection, telephone (507) 284-2329 or toll free (866) 273-4681.

Where can I get more information about clinical trials?

You may call the NCI's Cancer Information Service at 1-800-4-CANCER (1-800-422-6237) or TTY: 1-800-332-8615

Visit the NCI's Web sites: Cancer Trials: comprehensive clinical trials information http://cancertrials.nci.nih.gov

CancerNet™: accurate cancer information including PDQ http://cancernet.nci.nih.gov

Authorization To Use And Disclose Protected Health Information

Your privacy is important to us, and we want to protect it as much as possible. By signing this form, you authorize Mayo Clinic Rochester and the investigators to use and disclose any information created or collected in the course of your participation in this research protocol. This information might be in different places, including your original medical record, but we will only disclose information that is related to this research protocol for the purposes listed below.

This information will be given out for the proper monitoring of the study, checking the accuracy of study data, analyzing the study data, and other purposes necessary for the proper conduct and reporting of this study. If some of the information is reported in published medical journals or scientific discussions, it will be done in a way that does not directly identify you.

The study data sent by the study doctor to the sponsor does not include your name, address, social security number, or other information that directly identifies you. Instead, the study doctor assigns a code number to the study data and may use your initials. Some study data sent to the sponsor may contain information that could be used (perhaps in combination with other information) to identify you (e.g., date of birth). If you have questions about the specific health information that will be sent to the sponsor, you should ask the study doctor.

This information may be given to other researchers in this study (including those at other institutions), representatives of the sponsor of the study, U. S. Army Medical Research and Material Command, or private, state or federal government parties or regulatory authorities (U.S. and other countries) responsible for overseeing this research. These may include the Food and Drug Administration, the Office for Human Research Protections, or other offices within the Department of Health and Human Services, and the Mayo Foundation Office for Human Research Protections or other Mayo groups involved in protecting research subjects.

If this information is given out to anyone outside of Mayo, the information may no longer be protected by federal privacy regulations and may be given out by the person or entity that receives the information. However, Mayo will take steps to help other parties understand the need to keep this information confidential.

This authorization lasts until the end of the study.

You may stop this authorization at any time by writing to the following address:

Mayo Foundation
Office for Human Research Protection
ATTN: Notice of Revocation of Authorization
200 1st Street SW
Rochester, MN 55905

If you stop authorization, Mayo may continue to use your information already collected as part of this study, but will not collect any new information.

If you do not sign this authorization, or later stop authorization, you may not be able to receive study treatment.

What Other Things Might the Sponsor do with Study Data?

In addition to the uses listed above, companies that sponsor studies often use study data for other purposes that are not part of the study. For example, the company might use the study data for research purposes to support the scientific objectives of the study described in this consent document, to learn more about the effects (good and bad) of any drug, device or treatment included in the study, to better understand the disease(s) included in the study, or to improve the design of future studies. Also, the company might share the study data with other companies it does business with. The company might do these things during the study, or after the study has ended, and would not have to ask for your permission to do so. The sponsor might still use study data, even after you stop your authorization, or the authorization expires, as long as the study data was collected before your authorization stopped or expired. The ways in which the study data could be used in the future may not be known now, so we can't give you the details.

A copy of this form will be placed in your medical record.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this research study.

_____	_____	_____
(Date / Time)	(Printed Name of Participant)	(Clinic Number)
	_____	
	(Signature of Participant)	
_____	_____	
(Date / Time)	(Printed Name of Individual Obtaining Consent)	
	_____	
	(Signature of Individual Obtaining Consent)	

From: IRB Full Blue Friday [irbfbfri@mayo.edu]
Sent: Monday, May 09, 2005 3:14 PM
To: Markovic, Svetomir N., M.D., Ph.D.
Cc: Perez, Edith A., M.D.; Pockaj, Barbara A., M.D.; Marschke, Robert F. Jr., M.D.; Colon-Otero, Gerardo, M.D.; Maples, William J., M.D.; Frytak, Stephen, M.D.; Rubin, Joseph, M.D.; Ingle, James N., M.D.; Letendre, Louis, M.D.; Creagan, Edward T., M.D.; Jett, James R., M.D.; Gallenberg, Mary M., M.D.; Richardson, Ronald L., M.D.; Long, Harry J., M.D.; Buckner, Jan C., M.D.; Camoriano, John K., M.D.; Fitch, Tom R., M.D.; Loprinzi, Charles L., M.D.; Hartmann, Lynn C., M.D.; Marks, Randolph S., M.D.; Pitot, Henry C., M.D.; Call, Timothy G., M.D.; Bible, Keith C., M.D., Ph.D.; Rivera, Candido E., M.D.; Okuno, Scott H., M.D.; Burch, Patrick A., M.D.; Adjei, Alex A., M.D., Ph.D.; Jatoi, Aminah, M.D.; Quevedo, J. F., M.D.; Alberts, Steven R., M.D.; Croghan, Gary A., M.D., Ph.D.; Galanis, Evanthia, M.D.; Hobday, Timothy J., M.D.; Roy, Vivek, M.D.; Tan, Winston, M.D.; Peethambaram, Prema P., M.D.; Gornet, Michael K., M.D.; Merchan, Jaime R., M.D.; Gray, Richard J., M.D.; Erlichman, Charles, M.D.; Tun, Han W., M.D.; Kaur, Judith S., M.D.; Goetz, Matthew P., M.D.; Johnson, Elizabeth A., M.D.; Moreno Aspitia, Alvaro, M.D.; Kogut, Heidi L.; Moynihan, Timothy J., M.D.; Haveman, Theresa M., P.A.; Wirk, Mona M., M.D.; Northfelt, Donald W., M.D.; Kim, George P., M.D.; Hogan, Thomas F., M.D.; Waitman, Kathryn R.; Jones, Kortni R.; Liffrig, Kathleen M.; Dodd, Lynn L.; Ryan, Debra L.; Milburn, Jane M.; Sumrall, Susan V.; Loserth, Linnea T.; Alberts, Steven R., M.D.; sdlirb01@exsdl.mayo.edu; jaxirb@exjax.mayo.edu; resadm@exjax.mayo.edu; Earle, John D., M.D.
Subject: Minute Excerpt for IRB 782-05
The following is an excerpt from the minutes of the Full Board (Blue Friday) of the Mayo Foundation Institutional Review Boards meeting dated April 22, 2005:

Dr. Svetomir N. Markovic

The Board reviewed and unanimously (13-0) approved the protocol entitled MC0338, "MUC1/HER-2/neu Peptide-Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" from Dr. Svetomir N. Markovic as principal investigator (PI); Drs Edith A Perez and Barbara A Pockaj as co-principal investigators (CPI); Ms Jane M Milburn as protocol development coordinator (PDC); Mses Linnea Loserth and Susan V Sumrall as study coordinators (SC); Drs Alex Adjei, Steven R Alberts, Keith C Bible, Jan C Buckner, Patrick A Burch, Timothy G Call, Edward T Creagan, Gary A Croghan, Charles Erlichman, Evanthia Galanis, Mary M. Gallenberg, Matthew P Goetz, Lynn C. Hartmann, Timothy J. Hobday, James N. Ingle, Aminah Jatoi, Stephen Frytak, James R Jett, Judith S Kaur, Louis Letendre, Harry J. Long, Charles L. Loprinzi, Randolph S. Marks, Jaime R. Merchan, Timothy J. Moynihan, Scott H. Okuno, Prema P. Peethambaram, Henry C. Pitot, J. Fernando Quevedo, Ronald L. Richardson, Joseph Rubin, John K. Camoriano, Tom R. Fitch, Michael K. Gornet, Richard J. Gray, Thomas F. Hogan, Robert F. Marschke, Donald W. Northfelt, Gerardo Colon-Otero, Elizabeth A. Johnson, George P. Kim, William J. Maples, Alvaro Moreno-Aspitia, Candido Rivera, Vivek Roy, Winston Tan, Han Tun, Baldeep Wirk, Mses Theresa M Haveman, Kathryn R Waitman, Heidi L Kogut, and Kortni R Jones as study personnel obtaining Informed Consent (IC); and Mses Kathy Liffrig, Debra L. Ryan, and Lynn L. Dodd as study personnel Not obtaining Informed Consent (NIC). This approval is valid for exactly one year unless during the year the IRB determines that it is appropriate to halt or suspend the study earlier. The protocol was dated February 16, 2005. The Board noted IND# 12155 for the study drug, MUC 1 and HER-2/neu Peptides Combined with CpG Oligonucleotides (CpG 7909, Coley Pharmaceuticals), Granulocyte-Macrophage Colony-Stimulating Factor (Immunex) or both, Mixed with Montanide ISA-51 (Seppic) dated December 17, 2004. The Board noted the DSMB was appropriate for this study. A maximum of 45 adult participants with breast adenocarcinoma is approved for enrollment in this protocol at Mayo Clinic Rochester, Jacksonville, and Arizona. Up to 60 prospective participants may be screened in order to have 45 complete the study. In accordance with 45 CFR 46.306, the Board determined that prisoners are appropriate for enrollment in this study on a case-by-case basis with the

approval of the Medical Director at the Federal Medical Center, or by the responsible individual at other institutions. The Board also determined that all the criteria specified in 45 CFR 46.305(a) have been met. Funding will be accommodated within an existing grant cost center (#625-783-900 and 3T0522). The Board approved the participant consent form with additional revisions by the Board. The IRB office will provide the final approved consent form to the proponents. Drs Best and Hogan and Ms. Jones recused themselves and left the room. 782-05

Dr. J. K. Lobl, Chair
Ms. K. Larrabee, Specialist
Mayo Foundation Institutional Review Boards
Full Board (Blue Friday)

(20)

MAYO CLINIC

DAMD17-01-1-0318 Version IX 07/26/2005

Protocol Modification Request Form

Mayo Foundation Institutional Review Board (IRB)

PLEASE NOTE: This form must accompany ALL protocol modification requests. If consent form changes are required, an electronic copy of the revised consent form (with revisions indicated by track changes function) MUST be provided to IRB.

This form cannot be submitted electronically.

If hard copy documentation will be included with the request, please **print** this completed form, attach the supporting documentation, and mail ALL the materials together in hard copy to IRB, 201 Bldg, 4-60. It is extremely important that all materials (i.e. this form, revised consent, supporting materials) be sent together and that duplicate copies of materials are NOT sent to the IRB.

Today's date 08/30/05

1. IRB Number of study: **782-05**
2. Title: **MC0338 MUC1/HER-2/neu Peptide-Based Immunotherapeutic Vaccines for Breast Adenocarcinomas**
3. Principal Investigator: **Svetomir Markovic, M.D., Ph.D.** Pager # **4-6188**
4. Study Coordinator(s): **Jane Milburn, PDC** Pager # **6-8826** Fax # **4-5280**
5. Mark one of the following: ☒ Investigator-initiated changes ☐ Sponsor-initiated changes

   Amendment name and number (if any): **communication prior to activation** Date of amendment (if applicable): _____
6. a. Is this study also being done at another Mayo site under a separate IRB number? **Yes** ☐ **No** ☒

   b. *If yes*, indicate IRB number of the identical study/studies here: _____

   *Reminder: Modification requests for identical studies with different IRB #'s must be reviewed by the IRB at the same time.
7. **Nature of Requested Modifications:** please check all applicable categories, and describe each change concisely and specifically in the middle column of the table below. For example, "addition of a liver biopsy on day 3" or "increase frequency of [drug] dosing schedule from once weekly to twice weekly".
   In addition, the justification for each requested modification must be specified in the corresponding column.

Do not write "See attached summary" as a replacement for either the listing of modifications or justifications. If this form is incomplete in any way, it may be returned to you for completion.

☒	Change in study design or procedures	List of Modifications **Exclude prisoners from the study population.**	Justification for each requested change **The follow-up study is too long to follow the prisoner population. The funding agency (Department of Defense Breast Cancer Research Program) is adamantly opposed to treating prisoners in this study. Please specify in the excerpt that prisoners are not allowed to be enrolled in this study.**
☐	Revisions to consent form *Revised consent form must be*	List of Modifications	Justification for each requested change

	submitted electronically to the IRB as indicated at the top of the form.		

The current consent form template is accessible at http://researchweb.mayo.edu/irb/consent_forms.html

☐	**Addition** of study personnel (please include full name including middle initial, and indicate role in the study)	*Please check this box if added personnel are replacing others being removed, and clarify all replacements being made, including role in the study:* ☐	
☒	**Removal** of study personnel (please include full name including middle initial, and indicate role in the study)	**Please see revised list of Study Personnel - see justification below.**	
☐	Other changes (editorial, administrative, funding, corrections/changes, etc)	List of Modifications	Justification for each requested change **The funding agency (Department of Defense) requested that the study personnel be limited to those physicians who will actually be enrolling the breast cancer patients.**

If the protocol involves use of the GCRC, a copy of this submission must be sent to GCRC, Domitilla 5.

A COPY OF THE FULL PROTOCOL (INCORPORATING REQUESTED CHANGES) IS REQUIRED FOR IRB REVIEW. THESE REQUESTS WILL BE REVIEWED BY THE APPROPRIATE BOARD OR COMMITTEE.

S. Markovic — 9/12/05

Principal Investigator's Signature — Date

If you have questions, please refer to the online "Manual for Investigators Conducting Research Involving Humans" (http://researchweb.mayo.edu/irbmanual/), or call the IRB Office at 4-2329.

Mayo Clinic Cancer Center Study Personnel Attachment for *#MC0338-*
This listing is to include ALL key personnel for this trial, including those previously listed on the IRB application (aka "Summary of Information...")

Principal Investigator (PI) and Co-Principal Investigators (CPI)	Co-Investigators (CI)	Other Study Personnel to Obtain Informed Consent (IC)		Other Study Personnel Who Will Not Obtain Consent (NIC)	Other Personnel (Code as noted)
Rochester					
1. Svetomir N. Markovic, M.D., Ph.D.	*Not Applicable*	2. Alex Adjei, M.D. 3. Steven R Alberts, M.D. ~~4. Keith C. Bible, M.D.~~ ~~5. Jan C. Buckner, M.D.~~ ~~6. Patriek A. Burch, M.D.~~ ~~7. Timothy G. Call, M.D.~~ ~~8. Edward T. Creagan, M.D.~~ ~~9. Gary A. Croghan, M.D.~~ ~~10. Charles Erlichman, M.D.~~ ~~11. Evanthia Galanis, M.D.~~ ~~12. Mary M. Gallenberg, M.D.~~ 13. ~~Matthew P. Goetz, M.D.~~ 14. Lynn C. Hartmann, M.D. ~~15. Timothy J. Hobday, M.D.~~ 16. James N. Ingle, M.D. 17. Aminah Jatoi, M.D.	~~18. James R. Jett, M.D.~~ 19. Judith S. Kaur, M.D. ~~20. Louis Letendre, M.D.~~ 21. Harry J. Long, M.D. 22. Charles L. Loprinzi, M.D. ~~23. Randolph S. Marks, M.D.~~ ~~24. Jaime R. Merchan, M.D.~~ ~~25. Julian R. Molina, M.D.~~ 26. Timothy J. Moynihan, M.D. ~~27. Scott H. Okuno, M.D.~~ 28. Prema P. Peethambaram, M.D. ~~29. Henry C. Pitot, M.D.~~ ~~30. J. Fernando Quevedo, M.D.~~ ~~31. Ravi D. Rao, M.D.~~ ~~32. Ronald L. Richardson, M.D.~~ ~~33. Joseph Rubin, M.D.~~	34. Kathy Liffrig	35. Jane Milburn (PDC)
Scottsdale					
36. Barbara A. Pockaj, M.D. (CPI)		37. John K. Camoriano, M.D. 38. Tom R. Fitch, M.D. 39. Michael K. Gornet, M.D. ~~40. Richard J. Gray, M.D.~~ ~~41. Theresa M. Haveman, PA-C~~ 42. ~~Thomas F. Hogan, M.D.~~	~~43. Robert F. Marschke, Jr., M.D.~~ 44. Donald W. Northfelt, M.D. ~~45. Kathryn R. Waitman, NP~~ 46. Heidi L. Kogut (IC) 47. Kortni R. Jones (IC)	48. Debra L. Ryan 49. Lynn L. Dodd	50. Linnea Loserth (Study Coordinator)
Jacksonville					
51. Edith A. Perez, M.D. (CPI)		52. Gerardo Colon-Otero, M.D. ~~53. Elizabeth A. Johnson, M.D.~~ ~~54. George P. Kim, M.D.~~ ~~55. William J. Maples, M.D.~~ 56. Alvaro Moreno-Aspitia, M.D.	~~57. Candido Rivera, M.D.~~ 58. Vivek Roy, M.D. 59. Winston Tan, M.D. ~~60. Han Tun, M.D.~~ 61. ~~Baldeep Wirk, M.D.~~		62. Susan V. Sumrall, RN

Mayo Clinic Cancer Center

Investigator's Brochure

MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines For Breast Adenocarcinomas

Author: Svetomir N. Markovic, M.D., Ph.D.

Document Type: Investigator's Brochure

Edition Number: Edition 2 (dated September 13, 2005)

Release Date: September 13, 2005

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2. Summary

The currently proposed clinical study incorporates the use of a breast cancer vaccine consisting of three peptides (MUC1 and two HER-2) co-emulsified in Montanide ISA-51 with either granulocyte-macrophage colony stimulating factor (GM-CSF) or CpG-ODN administered to patients with previously treated, high risk of relapse breast adenocarcinoma. The goals of the study are to identify the safety and toxicity profile of the vaccine preparations as well as ascertain their immunological efficacy (ability to generate a specific immune response).

The rationale for the use of this specific vaccine preparation stem from published data describing the immune-adjuvant properties of Montanide ISA-51 combined with either GM-CSF or CpG-ODN or both. Our own preliminary data demonstrates the immunological efficacy of such adjuvants for peptide vaccines in mice. The choice of the vaccine peptides is based on their immunodominance and reactivity with HLA-A2 class I (MUC1 and HER-2) and class II (HER-2) molecules relevant for the generation of both cytotoxic and helper T cell immune responses respectively.

The expected toxicity to patients taking part in this study is minimal. Similar studies using different peptides have reported toxicities ranging from local skin reactions at the site of vaccine administration to transient systemic flu-like symptoms easily managed with non-pharmacologic interventions. We do not expect any additional toxicities in our study.

3. Introduction

Breast cancer is diagnosed in 200,000 individuals in the United States annually and contributes to approximately 40,000 deaths each year. For tumors confined to the breast, surgical removal provides a good prognosis. However, primary tumor that metastasizes to distant sites, such as lymph nodes, lungs, liver and brain, correlates with a poor prognosis. Patients with advanced stage breast cancer are at high risk of relapse. Complications from metastatic disease are the leading causes of cancer-related deaths. Novel adjuvant strategies, such as breast cancer specific vaccines, are being considered as a clinical intervention that may reduce the chance of recurrence.

In recent years there has been great interest in the development of these cancer vaccines, which are designed to immunize individuals to antigens present on tumors. Cancer vaccines are a non-toxic therapy, which have been shown in several melanoma trials to have the potential of controlling disease and prolonging survival because tumors can be surgically removed and there is often a long period of time before the tumor recurs at metastatic sites, cancer vaccines have been proposed as an optimal therapy that could prolong the time to recurrence. This optimal opportunity of immunization in the situation of minimal residual disease has rarely been tested, however, as most vaccines have been given to patients with large tumor burden after the failure of standard therapies in Phase I and Phase II trials. Recently, several groups have addressed the use of adjuvant immunotherapy following complete surgical resection [1]. Data from these studies are not yet complete.

The past two decades in tumor immunology have lead to the discovery of specific tumor antigens that have been shown in preclinical studies to elicit tumor-specific immunity and establish long term memory without autoimmunity. For breast cancer, vaccines composed of epitopes derived of MUC1, HER-2/neu, MAGE3, CEA have been studied and shown to be immunogenic without causing autoimmunity [2-5].

It is now clear that tumor antigens are presented in the context of specific class I and Class II HLA molecules. Class I presentation, in the presence of appropriate co-stimulation, is thought to stimulate a cytolytic $CD8^+$ T cell response, while antigen

presentation in the context of Class II molecules stimulates a $CD4^+$ helper T cell response [6].

One approach for the development of a cancer vaccine is the use of tumor associated synthetic antigens for immunologic priming. Because specific peptides are ubiquitous in tumors of the same histologic type, identical peptide vaccines may be employed in allogeneic hosts bearing the same tumor histology. Additionally, the use of single peptides for immunization limits the potential induction of undesired autoimmunity [7-9]. Recent developments in the use of soluble MHC Class I/peptide tetramers and elispot technology have enabled rapid characterization of epitope-specific CTL responses [10, 11]. In addition to being well-explored and understood, many of these antigens are shared tumor antigens. Vaccines that are composed of these antigens can be developed for use in a large number of patients. The primary limitations to peptide based vaccine strategies are haplotype restriction, potential for degradation, and uncertainty regarding which peptides, used alone or in combination, are the most immunogenic [12, 13]. This study is designed to test these uncertainties.

One attractive and broadly applicable target for immunotherapeutic strategies is the MUC1 tumor antigen. MUC1, a cell-associated mucin, is expressed on the cell surface of many epithelial malignancies as well as by hematological malignancies [14-17]. These include multiple myeloma (92%) and acute myelogenous leukemia (67%) [18]. Greater than 90% of breast carcinomas express MUC1; high levels are also found in adenocarcinomas originating from most tissues [14, 16]. MUC1 expression is greatly up-regulated on tumors (reviewed in Gendler [19]). Expression on tumors is no longer apical, but it is found all around the cell surface and in the cytoplasm. In addition, glycosylation on tumor-synthesized MUC1 is aberrant, with greater exposure of the peptide core than is found in normal tissues. MUC1 has long been an interesting target molecule for immunotherapeutic strategies, given its high level and ubiquitous expression. Patients with tumors, especially with breast, pancreas and ovarian tumors, have exhibited immune responses to MUC1 with the presence of antibodies and T cells specific for MUC1 detected in about 10% of individuals. An HLA unrestricted T cell response among cancer patients has also been described [20-23]. There is increasing evidence from murine and human studies that MHC-restricted T cells can be induced in mice and humans after immunization with the MUC1 peptide or MUC1 antigenic epitopes [24-32]. Importantly, there have been reports of two HLA-A2 binding peptides derived from the MUC1 protein [33]. One of the peptides is from the tandem repeat sequence of MUC1 and the second peptide is from the signal sequence. MUC1-specific cytotoxic T cells (CTLs) have been induced in T cells from healthy donors following *in vitro* immunization using peptide-pulsed dendritic cells. MUC1-specific CTLs have also been induced *in vivo* after vaccination of breast and ovarian cancer patients with peptide-pulsed DCs [18].

A second candidate for peptide-based immunotherapy is HER-2/neu, the gene product of the erbB2/neu protooncogene. HER-2/neu is overexpressed in approximately 30% of breast cancer patients. HER-2/neu is also expressed by multiple types of tumors, including ovarian, lung, colon, pancreas and gastric tumors [34-36]. HER-2/neu has particular relevance, as it is expressed at high levels in early *in situ* lesions in breast carcinoma [37]. Thus, it is a target for early disease. Immunologic responses to HER-2/neu have been detected in a minority of patients with advanced stage breast and ovarian cancer, including antibodies, T helper and CD8 responses [38, 39]. Several HLA-class I binding peptides have been previously identified. A novel HLA-A2.1 binding peptide from the HER-2/neu extracellular domain [HER-$2(9_{435})$] was recently identified [40]. This peptide (ILHNGAYSL) bound to HLA-A2.1 with intermediate affinity (IC50 74.6 nM). The HER-$2(9_{435})$ epitope was tested using an *in vitro* immunization protocol and

found to elicit CTLs that killed peptide-sensitized target cells. The CTLs elicited also recognized the HER-2/neu antigens, as it specifically killed tumor cells expressing the HLA-A2.1 and HER-2/neu antigens (see below in preliminary data). Furthermore, recognition of the tumor cell targets was significantly inhibited by unlabeled (cold) targets pulsed with HER-2(9_{435}), but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (see below). Thus, the CTLs induced by HER-2(9_{435}) are antigen specific.

A potential limiting factor for peptide based immunotherapy is related to a defined antigenic repertoire which is HLA restricted. This factor, inherent to all peptide-based approaches, restricts patient access. Additionally, because individual peptides only have the potential to induce epitope-specific CTL, the vast majority of potential tumor antigens are not targeted. In this setting, tumor down regulation of individual antigens or HLA epitopes promotes immune evasion. Recent evidence, however, suggests that this problem of epitope restriction may not be as physiologically important as was previously postulated. Specifically, it has now been clearly demonstrated that a T cell response induced against one epitope can stimulate CTL response to other target epitopes through a mechanism termed epitope spreading [3, 41, 42]. Using an experimental autoimmune encephalitis model, Vanderlugt et al. have demonstrated that disease progression is associated with the development of epitope-specific helper T cells, which are distinct from those initiating the disease. Transfer of secondary $CD4^+$ cells to naïve mice induces the disease phenotype and the disease is abrogated by blocking the secondary T cell response even though the primary T cell response remains intact [43, 44]. Disis demonstrated epitope spreading in 84% of patients vaccinated with HER-2/neu peptides, reflecting the initiation of an endogenous immune response. The immunity persisted after active immunizations ended [3]. These data suggest that peptide based approaches to cancer immunotherapy may indirectly stimulate multiple tumor reactive CTL against minor antigens in the presence of residual tumor. Based on this concept, the current study is designed as a therapeutic approach, with peptide epitope selection designed to enhance the number of potential candidates.

In addition to class I epitopes, immunogenic HLA-DR restricted class II epitopes have been defined for HER-2/neu. $CD4^+$ helper T lymphocytes (T_H) responses play an essential role in immunologically mediated anti-tumor immunity [45]. T_H lymphocytes provide CTLs with growth-stimulating cytokines, prime/activate DCs to effectively present antigen to naive CTL precursors [46-48] and they are important in the development of immune memory [49-51]. The development of IgG antibodies to HER-2/neu and the identification of $CD4^+$ T cells that secrete cytokines in response to HER-2/neu peptides or recombinant HER-2/neu protein suggest responses to helper T cells [52-57]. A promiscuous MHC class II T_H epitope has been identified for the HER-2/neu antigen (HER-2_{883}). T cell responses are restricted by HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53 [58]. Peptide-induced T cells were effective in recognizing naturally processed HER-2/neu protein. The peptide HER-2_{883}, (KVPIKWMALESILRRRF), which was selected by computer algorithm, was tested for its capacity to stimulate $CD4^+$ T cells isolated from four healthy, MHC-typed individuals (DR1/11, DR1/13, DR4/15, DR7/17) in primary *in vitro* culture using peptide pulsed autologous DCs. T cells that proliferated were found to react with peptide and recombinant HER-2/neu intracellular domain protein presented by autologous DCs (see below). These results, showing reactivity with recombinant protein, suggest that HER-2_{883} is naturally processed, as the peptide stimulated T cells react with DCs primed with recombinant protein. Clearly, HER-2_{883} is a naturally processed peptide epitope and is promiscuous for multiple HLA-DR epitopes, making it an ideal candidate for therapeutic applications.

Because of the expression of MUC1 and HER-2/neu in multiple cancers, the development of this peptide-based immunotherapy can potentially impact the treatment of multiple disease entities, not only adenocarcinomas but hematopoietic malignancies as well. There is considerable interest in the use of the MUC1 peptide vaccination for treatment of multiple myeloma following transplant when there is minimal residual disease prior to remission.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a commercially available cytokine currently used in patients undergoing chemotherapy to shorten the duration of post-chemotherapy neutropenia. Recently published evidence also suggests that GM-CSF may play a role as an immune adjuvant [59, 60]. The following observations illustrate the mechanisms by which GM-CSF can potentiate the immunogenicity of an antigen: 1) GM-CSF is a key mediator of dendritic cell (DC) maturation and function [61]; 2) GM-CSF increases surface expression of class I and II MHC molecules as well as co-stimulatory molecules of dendritic cells *in vitro [61]*; 3) GM-CSF enhances antibody responses to known immunogens *in vivo* [62]; 4) tumor cells transfected with genes encoding/expressing GM-CSF are able to induce long lasting, specific anti-tumor immune responses *in vivo* [63]; 5) GM-CSF encapsulated in biodegradable microspheres mixed with whole tumor cells resulted in systemic anti-tumor immune responses comparable to those of GM-CSF transfected tumor cells [64]. Therefore, addition of GM-CSF to an oligopeptide antigen may substantially enhance its immunogenicity.

In an attempt to optimally enhance the immunogenicity of the peptides we will deliver the antigens and GM-CSF emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA-51). This delivery mechanism should be comparable to a previously demonstrated delivery mechanism utilizing GM-CSF suspended in microspheres and mixed with tumor cells (antigens). We hypothesize that the emulsified GM-CSF in close proximity to tumor antigen peptides will substantially enhance their immunogenicity. This proximity of antigen and GM-CSF seems to be necessary for the adjuvant effect of GM-CSF, as systemic administration of equivalent doses in animal models has not demonstrated adjuvant activity. Also, the adjuvant/local inflammatory properties of IFA may play a role in attracting antigen presenting cells to the site of injection [53]. We have preliminary data demonstrating the plausibility of such a mechanism.

Preliminary data demonstrating the feasibility of this approach already exists. Rosenberg and investigators published effective generation of peptide-specific T cells in melanoma patients immunized with peptides derived from gp100 [65]. Despite the demonstration of a specific immune response, no clinical responses were detected. Addition of systemic GM-CSF resulted in more pronounced CTL and delayed type hypersensitivity reactions and in a few cases objective tumor regressions. Salgaller et al. utilized a peptide derived from the gp100 epitope suspended in IFA and demonstrated generation of specific T cell responses to the peptide in melanoma patients [66]. Both studies suggest that increased immunogenicity of the peptide antigens leads to a more pronounced T cell response, which in some cases results in a clinically relevant anti-tumor effect. In the proposed study, we will combine the immunoadjuvant effects of both IFA and GM-CSF with the goal of increasing the immunogenicity of the MUC1 and HER-2/neu immunodominant peptides.

Preliminary observations in an ongoing clinical study (MC9973) utilizing HLA-A2 specific melanoma differentiation antigen peptide vaccines in which the peptide is emulsified in a suspension of IFA and GM-CSF is demonstrating enhanced skin reactions if peptide emulsified in IFA is administered in the presence of GM-CSF. A dose of 50 μg of GM-CSF in the presence of IFA and peptide results in extensive local

skin reactions as well as evidence of a clinical response in one of seven patients thus far. No changes in the numbers of peptide specific CTLs were observed. However, a recent publication demonstrated superior numbers of vaccine specific CTLs generated in a peptide vaccine utilizing 225ug of GM-CSF in IFA) [67]. This would suggest a dose/response relationship of GM-CSF and anti-peptide vaccine CTL frequencies as determined by ELISPOT and tetramer assays. Therefore, in the current trial we propose to use 225ug of GM-CSF suspended in IFA (montanide ISA-51).

Therapeutic properties of bacteria in the treatment of malignant diseases (i.e. Coley's toxin) is an observation that has permeated the oncology literature for almost a century. More recently, it has been demonstrated that bacterial DNA possesses unique immunomodulatory features of potential utility in cancer therapy. Specifically, unmethylated CpG are able to stimulate NK cells and B cells. Furthermore, synthetic oligodeoxynucleotide (ODN) constructs containing unmethylated CpG motifs (CpG-ODN) were able to activate dendritic cells (DC) enhancing their antigen processing/presentation properties and stimulating production of Th1 cytokines necessary for CTL immune responses. Thus, CpG ODN appeared to function as an immune adjuvant. Several preclinical and clinical works illustrate the ability of CpG-ODN to function as a potent immune adjuvant for various forms of vaccines. One of the more interesting works, pertinent to this study, demonstrates the ability of CpG ODN to induce CTLs against a peptide vaccine when administered in conjunction with incomplete Freund's adjuvant (IFA) [68]. These authors used a MART-1/Melan-A_{26-35} peptide emulsified in IFA with or without the addition of 50ug of CpG ODN to immunize human D^b (HHD) A2 transgenic mice. Their data suggest superior anti-peptide immunization in the CpG-ODN immunized group as determined by the frequency of tetramer positive CTLs. Our own data support these findings demonstrating superior immunization efficacy of IFA+CpG-ODN with ova peptide of C57BL/6 mice when compared to either IFA+peptide or complete Freund's adjuvant (CFA) + peptide (data not shown). An additional benefit to the CpG-ODN adjuvant is that it has been shown to be especially good at enhancing cellular and humoral immunity and promoting a Th1-type of response in older mice [69]. The population that develops cancer is mainly older individuals, thus the CpG-ODN adjuvant may be particularly relevant for this trial. Based on preclinical data suggesting the potent immune adjuvant properties of CpG co-emulsified with peptides in IFA, we elected to test the efficacy of CpG-ODN in the setting of a peptide vaccine immunization in this clinical trial. The dose of CpG-ODN that we decided to use in this study is 2mg/vaccine. The dose is based on published data demonstrating a direct dose-dependent relationship of CpG-ODN (0.125 –1.0 mg) and magnitude of measured immune responses (HepB vaccine adjuvant Halperin [70]. This is well below the highest tested doses of 20mg/week. Based on these observations we feel that the 2mg dose is a reasonable starting point for a CpG-ODN adjuvant suspended in Montanide ISA-51 alone or in combination with GM-CSF.

4. Physical/chemical properties and formulation of vaccine components

4.1. MUC-1 (STAPPVHNV)

4.1.1. Other Names: epithelial membrane antigen (EMA), polymorphic epithelial antigen (PEM), DF3 antigen, Ca1, MAM-6, H23, episialin.
4.1.2. Active ingredient formula: $C_{40}H_{64}N_{12}O_{13}$
4.1.3. Molecular weight: 921.0
4.1.4. Appearance: white, amorphous powder.
4.1.5. Formulation and Storage: Samples will be vialed (glass vials with Teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at -20°C until use.
4.1.6. Drug Procurement and Accountability: purchased from Clinalfa

4.2. HER-2 Peptide-1 (ILHNGAYSL)

4.2.1. Other Names: erbB2, neu
4.2.2. Active ingredient formula:C45H70N12O13
4.2.3. Molecular weight: 987.1
4.2.4. Appearance: white amorphus powder
4.2.5. Formulation and Storage: Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at -20°C until use.
4.2.6. Drug Procurement and Accountability: purchased from Clinalfa

4.3. HER-2 Peptide-2 (KVPIKWMALESILRRRF)

4.3.1. Other Names: erbB2, neu
4.3.2. Active ingredient formula: C100H167N29O21S1
4.3.3. Molecular weight 2143.7
4.3.4. Appearance: white, amorphous powder
4.3.5. Formulation and Storage: Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at -20°C until use.
4.3.6. Drug Procurement and Accountability: purchased from Clinalfa

4.4. Montanide ISA-51 Adjuvant [MONTAN]

4.4.1. Formulation and Storage: Montanide ISA-51 is an oil-based adjuvant product similar to Incomplete Freund's Adjuvant. Which when mixed with a water-based solution on 1:1 w/w ration, forms a water-in-oil emulsion. It consists of highly purified oil, Drakol VR, and a surfactant, mannide oleate. Montanide ISA-51 is manufactured by Sepic, Inc., and is provided in amber glass ampoules containing 3 mL of the solution. Montanide ISA-51 will be purchased from Seppic Inc.
4.4.2. Storage and Stability: the solution is stored at controlled room temperature. Exposure to cold temperatures may result in a clouded solution, which should be discarded. An expiration date is printed on the ampoule label.
4.4.3. Compatibilities/Incompatibilities: the oil may break down the rubber tip of the plunger on syringes; it is advisable to use a different syringe for each ampoule. Do not allow the Montanide ISA-51 to be in direct contact with the rubber tip of the plunger for more time than is necessary to withdraw the solution and inject it into the peptide vial. Fresh syringes will be needed to withdraw the emulsified vaccine from the vaccine vial. Once the emulsion is

made, there is less interaction of the oil directly with the rubber tip of the plunger.

4.4.4. Drug Procurement and Accountability: Montanide ISA-51 will be purchased from Seppic Inc. The Cancer Center Pharmacy Shared Resource will store the drug and maintain records of inventory and disposition of all agent received.

4.5. GM-CSF (sargramostim, Leukine®)

4.5.1. Preparation and Storage: Liquid (used in this study) is available in vials containing 500 mcg/mL (2.8 × 10 6 IU/mL) sargramostim. Carton of 5 multiple-dose vials; each vial contains 1 mL of preserved 500 mcg/mL LEUKINE Liquid (NDC 50419-050-30). LEUKINE should be refrigerated at 2-8°C (36-46°F). Do not freeze or shake. Do not use beyond the expiration date printed on the vial.

4.5.2. Drug Procurement: Leukine 500 mcg vials are available commercially. Drug will be purchased using study grant funds (i.e. Patients will not be charged for GM-CSF).

4.6. CpG-7909 (Promune™)

4.6.1. Preparation and Storage: CpG-7909 is formulated as a sterile phosphate buffered saline solution (5mg/mL) stabile for parenteral administration. The sterile and pyrogen free solution contains no preservatives. Vials are intended for single entry: penetration of the vial's stopper should only be done once to maintain sterility. The drug product is packaged in clear, Type I USP glass vials with teflon-coated stopper closure and flip-caps. The drug product should be stored under refrigeration (2°-8°C). CpG-7909 is stable for at least one year if stored frozen.

4.6.2. Drug Procurement: to be purchased from Coley Pharmaceutical Group Inc.

5. Pre-clinical data

Preliminary data will be presented in multiple sections. First, we will provide data to support the choice of MUC1 and HER-2/erbB2 antigenic epitopes for this trial. Next, we will define our experience using peptides to stimulate tumor reactive T cells for cancer immunotherapy. Finally, we will discuss our experience with the immune adjuvants GM-CS and CpG-ODN. These preliminary data provide a strong foundation for the current proposal.

Identification of CTL Epitopes from MUC1

Using a computer analysis of the MUC1 amino acid sequence, two novel peptides were identified with a high binding probability to the HLA-A2 molecule [33]. Two peptides from MUC1 were identified; one from the tandem repeat M1.1 ($STAPPVHNV_{950-958}$) and one from the leader sequence M1.2 ($LLLLTVLTV_{12-20}$). The presence of the V in position 6 increases the binding of the M1.1 peptide to the HLA-A2 molecule. There is some variability in the tandem repeats in MUC1 and this sequence is found in the last tandem repeat. Cytotoxic T cells were induced from healthy donors by primary *in vitro* immunization using peptide-pulsed dendritic cells. The peptide-induced CTL lysed tumors endogenously expressing MUC1 in an antigen-specific and HLA-A2-restricted fashion.

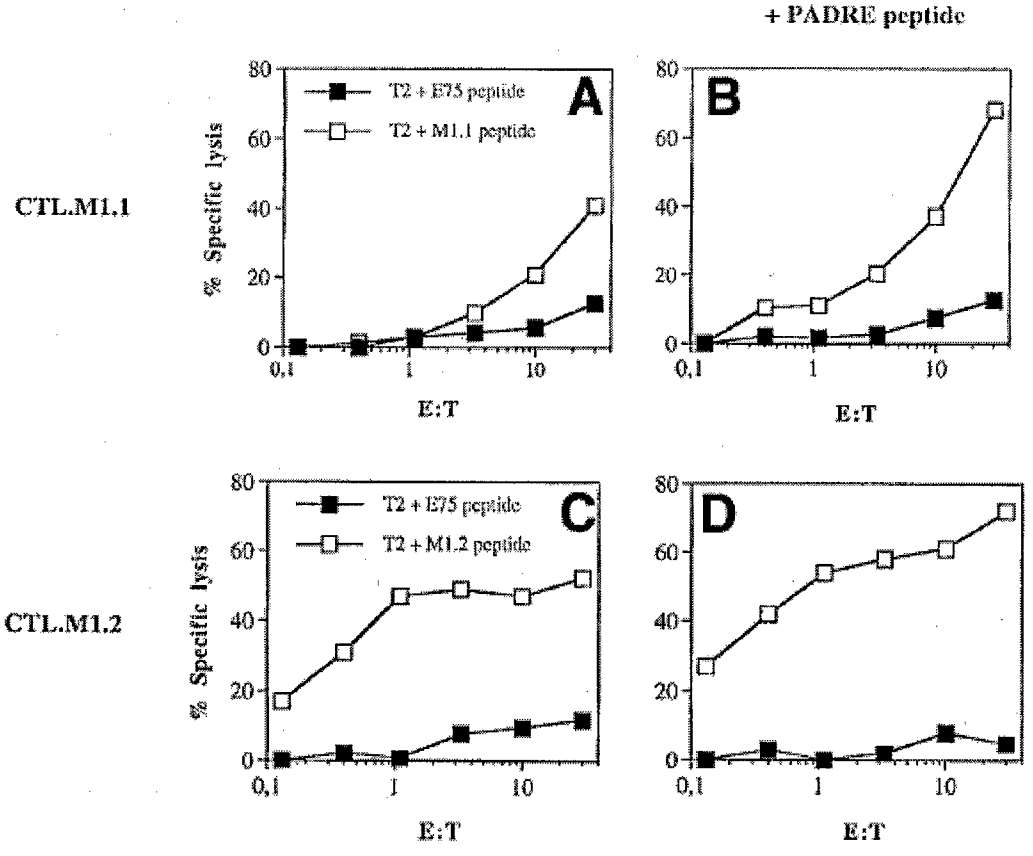

Figure 1. *Induction of CTL responses by peptide-pulsed dendritic cells. Adherent peripheral blood mononuclear cells were grown for 7 days with GM-CSF, IL-4, and TNF alpha. DCs pulsed with the synthetic peptides derived from the MUC1 protein (M1.1 and M 1.2) were used to induce a CTL response in vitro. In addition to the MUC1 peptide DCs were incubated with the PAN-DR binding peptide PADRE as a T-helper epitope. Cytotoxic activity of induced CTL was determined in a standard ^{51}Cr-release assay using T2 cells as targets pulsed for 2 hours with 50 µg of the cognate (open symbols) or irrelevant HER-2/neu protein-derived protein derived E75 peptide (solid symbols). (data reproduced from Brossart 1999 [33])*

Next, the ability of the induced MUC1-specific CTL lines to lyse tumors expressing MUC1 was tested. MCF-7 cells that express MUC1 endogenously and are HLA-A2

positive were used as targets in a standard ^{51}Cr-release assay. The controls were SK-OV-3 cells, which express MUC1, but are HLA-A2 negative and the immortalized B cell line, Croft, which is A2 positive and was pulsed with MUC1 M1.1 or M1.2 peptides or the irrelevant HER-2/neu E75 peptide.

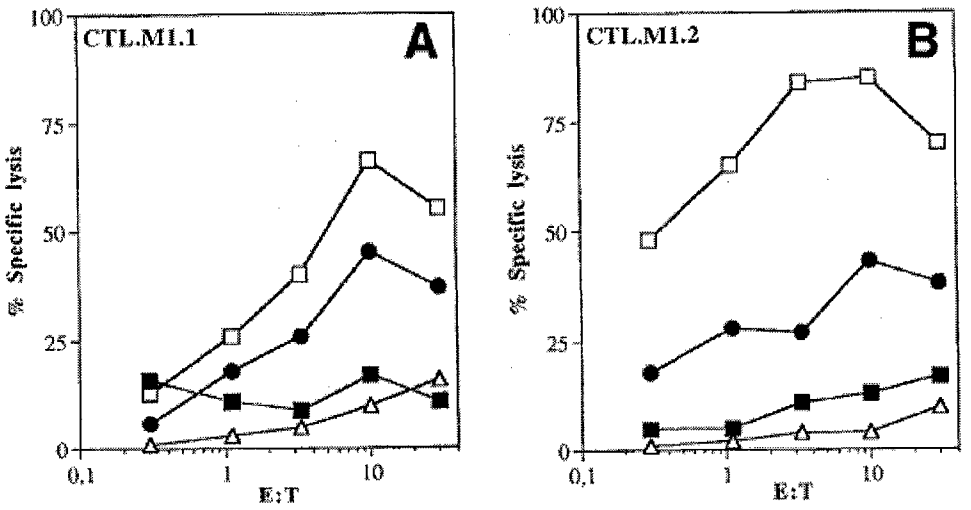

Figure 2. *Lysis of cancer cells endogenously expressing MUC1 by CTL.M1.1 (A) and CTL.M1.2 (B). Human breast cancer cell line MCF-7 (HLA-A2$^+$/MUC1$^+$), ovarian cancer cell line SK-OV-3 (HLA-A2$^-$/MUC1$^+$), and the immortalized B-cell line Croft (HLA-A2$^+$/MUC1$^-$) were used as targets in a standard ^{51}Cr-release assay. Croft cells were pulsed with the MUC1 peptides or an irrelevant HER-2/neu-derived peptide E75. (■) Croft + E75 peptide; (□) Croft + M1.1 (A) or M1.2 (B); (●)MCF-7; (Δ) SK-OV-3.*

We have chosen to use the M1.1 peptide based on the large amount of data on the response to the MUC1 tandem repeat peptide, both in the human situation as well as in the mouse. Obviously only the human data are relevant for the clinical trials. We will use a HER-2/neu helper epitope (see below, not the PADRE helper epitope)

In the case of HER-2/neu, we have identified a novel CTL epitope HER-2 (9_{435}), which bound HLA-A2.1 with intermediate affinity (IC_{50} 74.6 nM). The peptide identified is: ILHNGAYSL. The .221(A2.1) cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human lymphoblastoid cell line .221, was used as target (peptide loaded) to measure activity of HLA-A2.1 restricted CTL [71]. The CTLs elicited following in vitro stimulation effectively killed HLA-A2.1$^+$ tumor cells, showing that the antigen is appropriately processed by tumors (Fig. 3A). In addition, recognition of the tumor cell target was significantly inhibited by unlabeled (cold) target pulsed with HER-2 (9_{435}) peptide, but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (Fig. 3B).

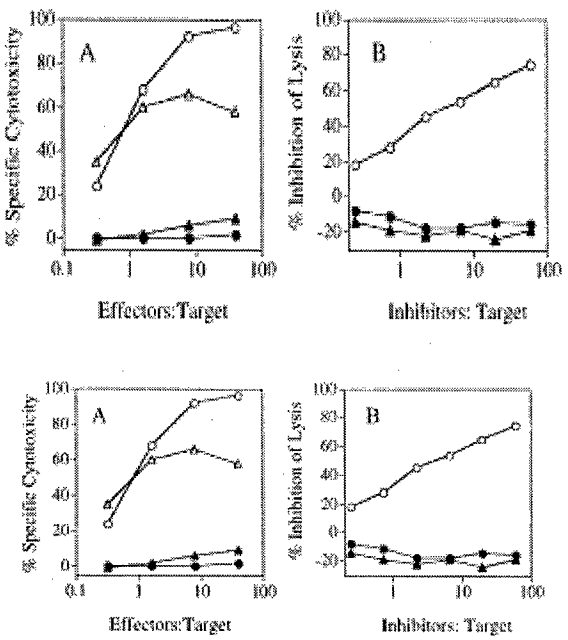

Figure 3. *HER-2(9_{435}) specific CTL can kill tumor cells. (A) HER-2(9_{435}) specific CTL were used as effector cells to test for the lysis of the following target cell lines: o, .221A2.1 pulsed with HER-2(9_{435}); ●, .221A2.1 without peptide; Δ, SW403 (colon CA, A2$^+$, HER-2/neu$^+$); ▲, HT-29 (colon ca, A2$^-$, HER-2/neu$^+$). (B): Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr labeled SW403 cells at an effectors/target ratio of 10:1 by the HER-2(9_{435}) specific CTL was blocked at various Inhibitors/Target ratios by the following cold targets: o, .221A2.1 pulsed with HER-2(9_{435}); ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); ●, .221A2.1 without peptide.*

In addition to the class I epitopes described above, we (EC) have defined a promiscuous MHC class II epitope for HER-2/neu using the algorithm tables published by Southwood et al. [58, 72]. The epitope identified is HER-2_{883} (KVPIKWMALESILRRRF). It is important to show that these peptides represent true T cell epitopes that are relevant for the development of tumor immunotherapy. For these experiments autologous PBMCs or DCs were used as APCs and recombinant DNA derived intracellular domain or extracellular domain protein fragments of HER-2/neu were used as a source of antigen. The data in Fig. 4 show that four HER-2_{883}-reactive T cell lines proliferated well to HER-2/neu intracellular domain protein, which encompasses the HER-2_{883} peptide but not to HER-2/neu extracellular domain (ECD), which lacks HER-2_{883}.

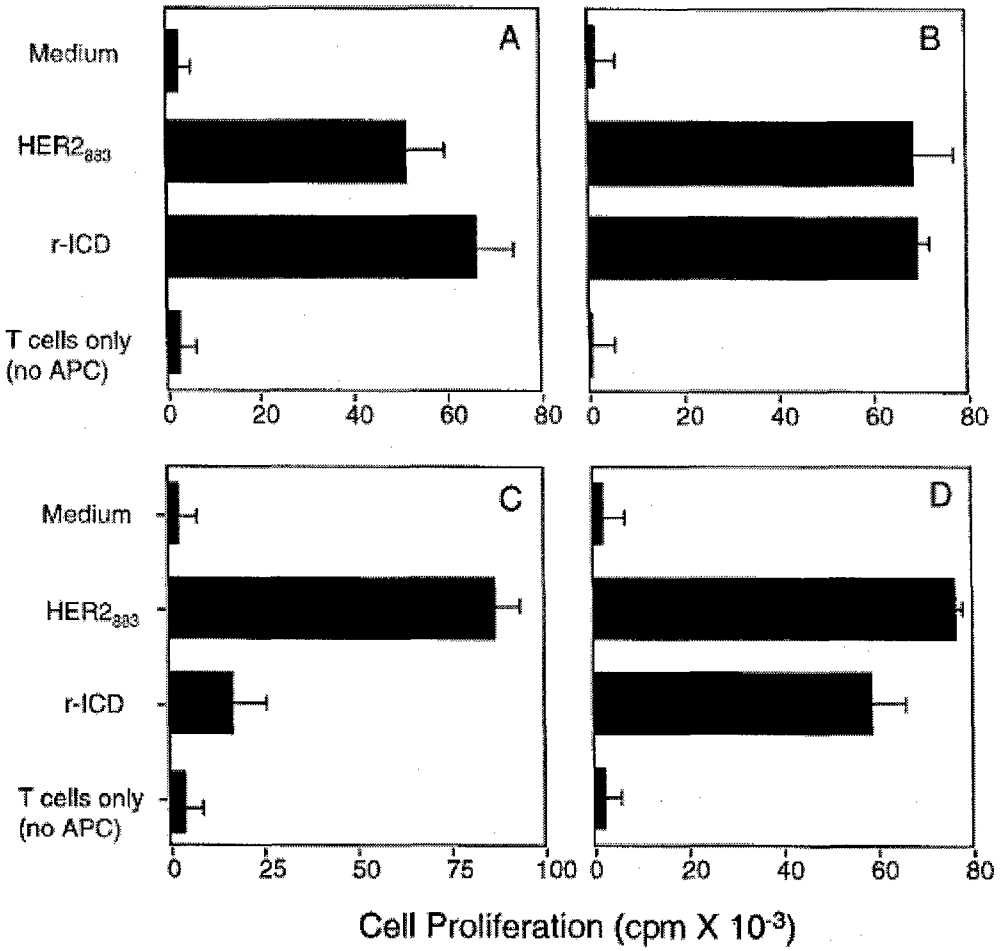

Figure 4. *HER-2_{883}-specific CD4+ T cells can recognize recombinant HER-2/neu intracellular domain (r-ICD) protein presented by autologous Dcs in the context of several HLA-DR alleles. The HER-2_{883}-reactive HTLs, TCL-7C (panel A, HLA-DR53 restricted), TCL-6D (panel B, HLA-DR4-restricted), a clone of TCL-1D (panel C, HLA-DR52-restricted), and TCL-1E (panel D. HLA-DR53 restricted), were tested for their capacity to proliferate to autologous DCs in the presence of HER-2_{883} peptide (2.5 mg/ml) or recombinant HER-2/neu recombinant ICD protein (10 mg/ml). No significant proliferative response was observed against HER-2/neu ECD protein (data not shown). Values shown are the means of triplicate determinations; bars, SD.*

Justification of vaccination strategy

Peptide dose (1000ug): Over the last several years there has been extensive debate over the optimal dose of peptide in a variety of peptide immunization cancer clinical trials. Peptide doses have ranged from 50ug to 2500ug in various studies. Currently, the largest peptide vaccine clinical trial (E4697) utilizes a peptide dose of 1000ug. There are several published studies evaluating peptide vaccine dose-responses [66, 73] suggesting that 1000ug of peptide would be a reasonable vaccine dose for phase I/II clinical testing.

GM-CSF suspended in Montanide ISA-51 as a vaccine adjuvant. The utility of GM-CSF suspended in montanide ISA-51 as an effective vaccine adjuvant has already been demonstrated in pre-clinical and clinical studies. Our own pre-clinical data (Fig 5) demonstrates a bell shaped dose-response curve for GM-CSF co-emulsified with 10ug of *ova* peptide in montanide ISA-51. Two weeks after immunization, the optimal dose of GM-CSF in the mouse model appears to be 100ug. In humans, Slingluff et al demonstrated successful peptide immunization using 225ug of GM-CSF suspended in montanide ISA-51 [67]. Up to 80% of treated patients demonstrated effective immunization with melanoma differentiation antigen peptides. Our clinical data using 10, 50, 75 and 100 ug of GM-CSF suspended with peptides in Montanide ISA-51 failed to demonstrate effective generation of anti-peptide CTLs. In view of these data, we felt that it was reasonable to utilize the same dose of GM-CSF used by Slingluff [67] (225ug) with our current set of peptides. If successful, further studies will be performed attempting to generate a dose-response curve of GM-CSF and immunization efficacy similar to that of the mouse model.

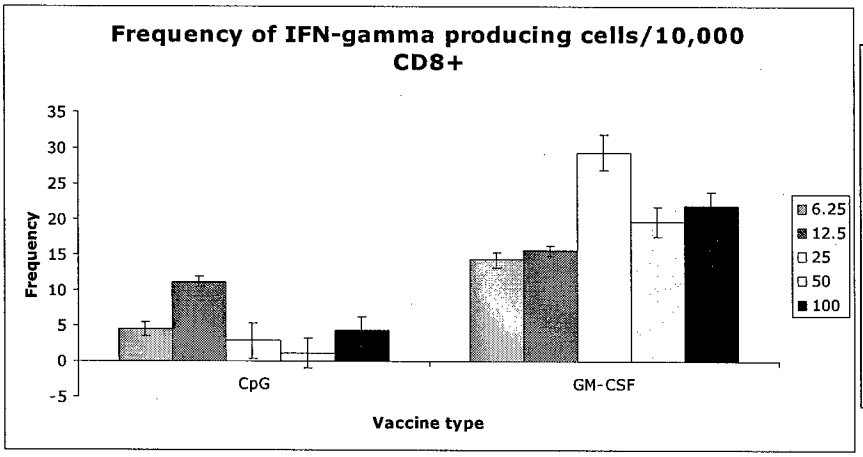

Fig. 5: C57BL6 mice (3 per group) were immunized with 10ug of ova peptide suspended in montanide ISA-51 and varying concentrations of CpG or GM-CSF. Represented are the frequencies of ova specific CTLs (IFN gamma ELISPOT) isolated from splenocytes on day 12 post immunization. Similar dose/response curves were observed in two other experiments.

CpG suspended in Montanide ISA-51 as vaccine adjuvant. As described in section 4.4.1, the co-emulsification of peptide antigens with CpG and Montanide ISA-51 is an effective means of generation of peptide specific CTLs in a pre-clinical model. Our own data confirm these findings using non-transgenic mice immunized with ova peptide co-suspended with CpG in Montanide ISA-51 (Fig. 5). The dose of CpG used in the current study was empirically selected based on the results of a phase I clinical trial utilizing CpG (abbreviated as ISS in Fig. 6 legend) as an immune adjuvant for hepatitis B vaccine immunization in healthy volunteers. In this study, volunteers were immunized with an intramuscular injection of hepatitis B vaccine (20ug) mixed with CpG in one of the following concentrations: 225ug, 650ug, 1000ug or 2250ug. A booster injection was administered 2 months later. Serologic data demonstrated (Fig 6) maximal immunization efficacy at CpG doses between 1000 and 2250ug. Based on these data suggesting a bell-shaped dose response curve for CpG (optimum may be between doses 1000ug and 2250ug) as well as our pre-clinical bell-shaped dose response curve, we elected to proceed with a CpG dose of 2000 ug.

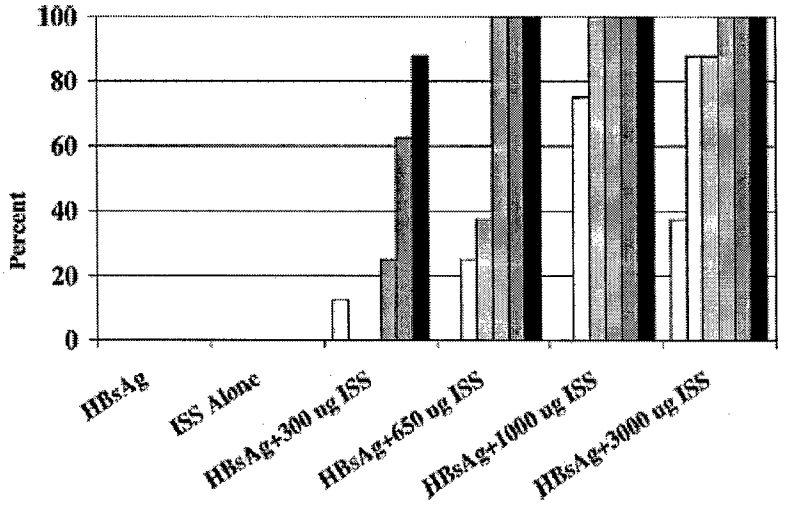

Figure 6: Proportion of participants achieving a protective antibody level (≥10mIU/mL) at various time points after immunization. Time points are (by increasing darkness of bar shade) 7 days after dose 1, 28 days after dose 1, 56 days after dose 1, 7 days after dose 2, 4 months after dose 2.

6. References

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DAMD17-01-1-0318

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

STATEMENT OF INVESTIGATOR

(TITLE 21, CODE OF FEDERAL REGULATIONS (CFR) PART 312)

(See instructions on reverse side.)

Form Approved: OMB No. 0910-0014.
Expiration Date: January 31, 2006.
See OMB Statement on Reverse.

NOTE: No investigator may participate in an investigation until he/she provides the sponsor with a completed, signed Statement of Investigator, Form FDA 1572 (21 CFR 312.53(c)).

1. NAME AND ADDRESS OF INVESTIGATOR

Svetomir N. Markovic, M.D., Ph.D.

Mayo Clinic in Rochester
200 First Street SW
Rochester, MN 55905

2. EDUCATION, TRAINING, AND EXPERIENCE THAT QUALIFIES THE INVESTIGATOR AS AN EXPERT IN THE CLINICAL INVESTIGATION OF THE DRUG FOR THE USE UNDER INVESTIGATION. ONE OF THE FOLLOWING IS ATTACHED.

☒ CURRICULUM VITAE ☐ OTHER STATEMENT OF QUALIFICATIONS

3. NAME AND ADDRESS OF ANY MEDICAL SCHOOL, HOSPITAL OR OTHER RESEARCH FACILITY WHERE THE CLINICAL INVESTIGATION(S) WILL BE CONDUCTED.

Mayo Clinic in Jacksonville, 4500 San Pablo Road, Jacksonville FL 32224
Mayo Clinic in Rochester, 200 First Street SW, Rochester, MN 55905
Mayo Clinic in Scottsdale, 13400 East Shea Boulevard, Scottsdale, Arizona 85259

4. NAME AND ADDRESS OF ANY CLINICAL LABORATORY FACILITIES TO BE USED IN THE STUDY.

Mayo Clinic in Jacksonville, Department of Laboratory Medicine and Pathology, 4500 San Pablo Road, Jacksonville FL 32224
Mayo Clinic in Rochester, Department of Laboratory Medicine and Pathology, 200 First Street SW, Rochester, MN 55905
Mayo Clinic in Scottsdale, Department of Laboratory Medicine/Pathology,13400 East Shea Boulevard, Scottsdale, Arizona 85259

5. NAME AND ADDRESS OF THE INSTITUTIONAL REVIEW BOARD (IRB) THAT IS RESPONSIBLE FOR REVIEW AND APPROVAL OF THE STUDY(IES).

Mayo Foundation Institutional Review Board
200 First Street SW
Rochester, MN 55905

6. NAMES OF THE SUBINVESTIGATORS *(e.g., research fellows, residents, associates)* WHO WILL BE ASSISTING THE INVESTIGATOR IN THE CONDUCT OF THE INVESTIGATION(S).

Mayo Clinic Rochester: Alex A. Adjei, M.D., Ph.D.; Steven R. Alberts, M.D.; Matthew P. Goetz, M.D.; Lynn C. Hartmann, M.D.; Timothy J. Hobday, M.D.; James N. Ingle, M.D.; Aminah Jatoi, M.D.; Judith S. Kaur, M.D.; Harry J. Long, M.D.; Charles L. Loprinzi, M.D.; Timothy J. Moynihan, M.D.; Prema P. Peethambaram, M.D.
Mayo Clinic Scottsdale: Barbara A. Pockaj, M.D.; John C. Camoriano, M.D.; Tom R. Fitch, M.D.; Michael K. Gomet, M.D.; Donald W. Northfelt, M.D.
Mayo Clinic Jacksonville: Edith Perez, M.D.; Gerardo Colon-Otero, M.D.; Alvaro Moreno-Aspitia, M.D.; Vivek Roy, M.D.; Winston Tan, M.D.

7. NAME AND CODE NUMBER, IF ANY, OF THE PROTOCOL(S) IN THE IND FOR THE STUDY(IES) TO BE CONDUCTED BY THE INVESTIGATOR.

MC0338 – MUC1/HER-s/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas

DAMD17-01-1-0318

8. ATTACH THE FOLLOWING CLINICAL PROTOCOL INFORMATION:

☐ FOR PHASE 1 INVESTIGATIONS, A GENERAL OUTLINE OF THE PLANNED INVESTIGATION INCLUDING THE ESTIMATED DURATION OF THE STUDY AND THE MAXIMUM NUMBER OF SUBJECTS THAT WILL BE INVOLVED.

☒ FOR PHASE 2 OR 3 INVESTIGATIONS, AN OUTLINE OF THE STUDY PROTOCOL INCLUDING AN APPROXIMATION OF THE NUMBER OF SUBJECTS TO BE TREATED WITH THE DRUG AND THE NUMBER TO BE EMPLOYED AS CONTROLS, IF ANY; THE CLINICAL USES TO BE INVESTIGATED; CHARACTERISTICS OF SUBJECTS BY AGE, SEX, AND CONDITION; THE KIND OF CLINICAL OBSERVATIONS AND LABORATORY TESTS TO BE CONDUCTED; THE ESTIMATED DURATION OF THE STUDY; AND COPIES OR A DESCRIPTION OF CASE REPORT FORMS TO BE USED.

9. COMMITMENTS:

I agree to conduct the study(ies) in accordance with the relevant, current protocol(s) and will only make changes in a protocol after notifying the sponsor, except when necessary to protect the safety, rights, or welfare of subjects.

I agree to personally conduct or supervise the described investigation(s).

I agree to inform any patients, or any persons used as controls, that the drugs are being used for investigational purposes and I will ensure that the requirements relating to obtaining informed consent in 21 CFR Part 50 and institutional review board (IRB) review and approval in 21 CFR Part 56 are met.

I agree to report to the sponsor adverse experiences that occur in the course of the investigation(s) in accordance with 21 CFR 312.64.

I have read and understand the information in the investigator's brochure, including the potential risks and side effects of the drug.

I agree to ensure that all associates, colleagues, and employees assisting in the conduct of the study(ies) are informed about their obligations in meeting the above commitments.

I agree to maintain adequate and accurate records in accordance with 21 CFR 312.62 and to make those records available for inspection in accordance with 21 CFR 312.68.

I will ensure that an IRB that complies with the requirements of 21 CFR Part 56 will be responsible for the initial and continuing review and approval of the clinical investigation. I also agree to promptly report to the IRB all changes in the research activity and all unanticipated problems involving risks to human subjects or others. Additionally, I will not make any changes in the research without IRB approval, except where necessary to eliminate apparent immediate hazards to human subjects.

I agree to comply with all other requirements regarding the obligations of clinical investigators and all other pertinent requirements in 21 CFR Part 312.

INSTRUCTIONS FOR COMPLETING FORM FDA 1572
STATEMENT OF INVESTIGATOR:

1. Complete all sections. Attach a separate page if additional space is needed.
2. Attach curriculum vitae or other statement of qualifications as described in Section 2.
3. Attach protocol outline as described in Section 8.
4. Sign and date below.
5. FORWARD THE COMPLETED FORM AND ATTACHMENTS TO THE SPONSOR. The sponsor will incorporate this information along with other technical data into an Investigational New Drug Application (IND).

10. SIGNATURE OF INVESTIGATOR	11. DATE
S. Markovic	9/14/05

(WARNING: A willfully false statement is a criminal offense. U.S.C. Title 18, Sec. 1001.)

Public reporting burden for this collection of information is estimated to average 100 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to:

Food and Drug Administration
CBER (HFM-99)
1401 Rockville Pike
Rockville, MD 20852-1448

Food and Drug Administration
CDER (HFD-94)
12229 Wilkins Avenue
Rockville, MD 20852

"An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number."

Please DO NOT RETURN this application to this address.

DAMD17-01-1-0318

From: Milburn, Jane M.
Sent: Tuesday, August 02, 2005 2:43 PM
To: Nelson, Carol J.
Subject: IRB Excerpt IRB 782-05 -MC0338 (IDB) Please file - In development
From: IRB Serious Adverse Events [mailto:irbserae@mayo.edu]
Sent: Tuesday, August 02, 2005 1:25 PM
To: Markovic, Svetomir N., M.D., Ph.D.
Cc: Perez, Edith A., M.D.; Pockaj, Barbara A., M.D.; Milburn, Jane M.; Sumrall, Susan V.; Loserth, Linnea T.; sdlirb01@exsdl.mayo.edu; jaxirb@exjax.mayo.edu; resadm@exjax.mayo.edu; Earle, John D., M.D.
Subject: Minute Excerpt for IRB 782-05

The following is an excerpt from the minutes of the Serious Adverse Events Board of the Mayo Foundation Institutional Review Boards meeting dated July 20, 2005:

Dr. Svetomir N. Markovic

The Board reviewed the revised Investigator's Brochure, Edition 1, dated May 31, 2005 for safety data, and unanimously (6-0) approved it for use in the study entitled MC0338, "MUC1/HER-2/neu Peptide-Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" from Dr. S. N. Markovic. No consent form revisions are required. 782-05

Dr. R. K. Pearson, Chair
Mr. W. F. Obritsch, Specialist
Mayo Foundation Institutional Review Boards
Serious Adverse Events Board

(75)

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville, MD 20852-1448

Our Reference: BB-IND 12155

December 21, 2004

Svetomir N. Markovic, M.D., Ph.D.
Associate Professor
Mayo Clinic
200 First Street, South West
Rochester, MN 55905

Dear Dr. Markovic:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND)**. The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

BB-IND #: 12155

SPONSOR: Svetomir N. Markovic, M.D., Ph.D.

PRODUCT NAME: MUC 1 and HER-2/neu Peptides Combined with CpG Oligonucleotides (CpG 7909, Coley Pharmaceuticals), Granulocyte – Macrophage Colony-Stimulating Factor (Immunex) or both, Mixed with Montanide ISA-51 (Seppic)

DATE OF SUBMISSION: December 1, 2004

DATE OF RECEIPT: December 17 2004

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file**. Please include three originals of all illustrations which do not reproduce well.

It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in

writing of the reasons for placing the IND on hold.

You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information. All serious, unexpected adverse experiences, as well as results from animal studies that suggest significant clinical risk, must be reported, in writing, to this Division and to all investigators within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

As required by the Food and Drug Modernization Act and the Best Pharmaceuticals for Children Act, you are responsible for registering certain clinical trials involving your drug product in the

Clinical Trials Data Bank http://clinicaltrials.gov/ and http://prsinfo.clinicaltrials.gov/. If your drug is intended for the treatment of a serious or life-threatening disease or condition and you are conducting clinical trials to test its effectiveness, then you must register these trials in the Data Bank. Although not required, we encourage you to register effectiveness trials for non-serious diseases or conditions as well as non-effectiveness trials for all diseases or conditions, whether or not they are serious or life-threatening. Additional information on registering your clinical trials, including the required and optional data elements and the FDA Draft Guidance for Industry, "Information Program on Clinical Trials for Serious or Life-Threatening Diseases and Conditions," is available at the Protocol Registration System (PRS) Information Site http://prsinfo.clinicaltrials.gov/.

Telephone inquiries concerning this IND should be made directly to me at (301) 827-5102. Correspondence regarding this file should be addressed as follows:

Food and Drug Administration
Center for Biologics Evaluation and Research
Attn: Office of Cellular, Tissue and Gene Therapies
Document Control Center, HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,

Myung-Joo Patricia Hong
Regulatory Project Manager
Regulatory Management Staff
Office of Cellular, Tissue and Gene Therapies
Center for Biologics Evaluation and Research

Enclosures (3): 21 CFR Part 312
21 CFR 50.20, 50.25
Information sheet on 21 CFR 25.24

MERCK

TERMS and CONDITIONS of SALE for CLINALFA® PRODUCTS

1. STANDARDS

Merck Biosciences AG CLINALFA® branded products comprise investigational parenteral medicinal products not "authorized for marketing" that must be used exclusively in human studies (clinical trials) that fully comply with the international ethical and scientific quality standards, as stated in ICH Guideline E6: Good Clinical Practice. CLINALFA® products are sold exclusively to professionals (physicians) who must provide adequate evidence (e.g. IND approval or equivalent, such as completed Product Disclaimer Agreement Form) of adherence to these standards, which must be in possession of Merck Biosciences AG before goods can be delivered.

CLINALFA® parenteral medicinal products are manufactured according to The Rules Governing Medicinal Products in the European Union, Volume 4: Good Manufacturing Practices, Medicinal Products for Human and Veterinary Use, Annex 1, using drug substances of the quality specified in the Contract of Sale.

2. PRICES and PAYMENT

The price of goods is exclusive of costs of shipping, insurance, any taxes, duties and other impositions, all of which shall be paid or borne by the buyer.

A 30% pre-payment shall be made within 30 days from the date of Contract of Sale for all orders with a value > USD 10'000. The goods shall be delivered and invoiced at the date(s) indicated in the Contract of Sale. Payment shall be made in full within 30 days from the date(s) of invoice. Prevention of delivery by any reason outside the power of Merck Biosciences AG, e.g. missing IND number, or change in the buyer's needs, does not relieve the buyer of meeting his liabilities.

3. CANCELLATION

The buyer may not cancel their purchase order without the written consent of Merck Biosciences AG, which if given shall be deemed to be on the express condition that the buyer shall indemnify Merck Biosciences AG against all loss, damage, claims or action arising out of such cancellation unless otherwise agreed in writing.

4. LIABILITY

Merck Biosciences AG will take no responsibility for the safety and efficacy of the products. The buyer acknowledges that it is purchasing the product for use in investigational clinical trials that are approved by the Institutional Review Board (IRB) according to the ICH Guideline E6: Good Clinical Practice. The IRB ensure the rights, safety, and well being of human subjects involved in the trial. Merck Biosciences AG takes no responsibility in this regard. The buyer shall not use or make otherwise available in any way whatsoever the product for other alternative human application. The buyer shall indemnify Merck Biosciences AG against all losses, damages, claims or actions arising out of the use of CLINALFA® products as investigational parenteral medicinal products, except where such losses or damages arise from a) the failure of CLINALFA® products to meet their specification, b) the negligent failure of Merck Biosciences AG to disclose relevant information in its possession regarding the safety of CLINALFA® products. Due to inherent imponderabilities, fulfillment of all specifications cannot be warranted with Customized Products. Any claim for compensation, e.g. for loss of drug substance confined, or for collateral damage resulting from non-compliance with this contract, is excluded with Customized Products.

5. GOVERNING LAW

These Terms and Conditions are deemed entered into in the country of Switzerland and will be governed and construed in all respects according to the laws of the country of Switzerland. Any litigation or other dispute resolution between the parties, relating to these Terms and Conditions shall take place in Sissach, Baselland, Switzerland. By placing an order for any CLINALFA® product the purchaser consents to personal jurisdiction under that venue.

vs040311

Merck Biosciences AG
Weidenmattweg 4
CH-4448 Läufelfingen
Phone 0041 62 285 25 25
Fax 0041 62 285 25 20
www.merckbiosciences.ch

DAMD17-01-1-0348

CERTIFICATE OF ANALYSIS for CLINALFA® PRODUCTS

PRODUCT NAME: **HER 2/neu (435-443)**
PRODUCT NUMBER: **C- S-242** BATCH NUMBER: **AC 0522**
ACTIVE INGREDIENT NAME: H-Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu-OH (ILHNGAYSL)
ACTIVE INGREDIENT FORMULA: C45 H70 N12 O13 MOLECULAR WEIGHT: 987.1

TESTS (Methods)	SPECIFICATIONS	RESULTS at RELEASE 9-Jul-04	RESULTS at RETEST
IDENTITY of Active Ingredient (coeleution, HPLC/UV)	Single Principal Peak	complies	
VIAL CONTENT of Active Ingredient (mean of N vials, HPLC/UV)	0.90 - 1.10 mg /N=6	1.08 mg /N=6	
UNIFORMITY of Vial Content (mean of N vials, HPLC/UV)	CV ≤ 5 % /N=6 (CV=Coefficient of Variation)	0.5 % /N=6	
ACTIVE INGREDIENT RELATED IMPURITIES (mean of N vials, HPLC/UV)	Total ≤ 5 % /N=6 Any ≤ 3 % /N=6	0.6 % /N=6 complies	
ACTIVE INGREDIENT PURITY (mean of N vials, HPLC/UV)	≥ 95 % /N=6	99.4 % /N=6	
STERILITY (thioglycollate & CASO, EP/USP membrane filtration method)	No evidence of microbial growth	complies	
PYROGENS (body temperature in rabbits, EP/USP method, i.v. dose: 0.03 mg /kg)	Summed response in 3 rabbits ≤ 1.15 °C	complies	

N. D. = NO DATA

NOTES:
Excipients: -
Soluble in 0.9 % NaCl
Vials sealed under N2.

Store at ≤ -18 °C, protect from light.
Estimated Expiry Date (Retest Date): 7-Jan-05

DATE: 16-Jul-04

A. Sauter, PhD
Director Quality Management

To be Used in Approved Clinical Trials Only!

Merck Biosciences AG
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DAMD17-01-1-0318

CERTIFICATE OF ANALYSIS for CLINALFA® ACTIVE INGREDIENTS

PRODUCT (API) NAME:	HER 2/neu (435-443)		
API NUMBER:	05-59-0438	API BATCH NUMMER: A 31046	MOL Wt.: 987.1
CHEMICAL NAME: (AA SEQUENCE)	H-Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu-OH (ILHNGAYSL)		FORMULA: C45 H70 N12 O13

TESTS	SPECIFICATIONS	RESULTS
Appearance	white to off white, amorphous powder	white, amorphous powder
Mass Spectrum	ES-MS: monoisotopic, non-protonated mass = 986.5 ± 1 Da (deduced from series of m/z signals)	986.6 Da
Amino Acids	Ala: 1.0 Asx: 1.0 Gly: 1.0 His: 1.0 Ile: 1.0 Leu: 2.0 Ser: 1.0 Tyr: 1.0 (standard values)	Ala: 1.1 Asx: 1.1 Gly: 1.1 His: 1.1 Ile: 1.0 Leu: 2.0 Ser: 1.1 Tyr: 1.1 (ADOPTED DATA)
Purity (HPLC/UV)	Impurities < 2 % Purity > 98 % (product related impurities)	99.8 %
Net Peptide Content	not specified (elementary N-analysis)	89.3 %
Water (GC)	not specified	6.5 %
Acetic Acid	not specified	2.6 %
Trifluoro Acetic Acid	< 1 %	< 0.1 %
Acetonitrile	< 400 ppm	< 30 ppm
INFORMATION	N. D. = NO DATA	**Store dry at ≤ -18 °C**

DATE: 7-Jun-04

A. Sauter, PhD
Director Quality Management

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DAMD17-01-1-0318

Labor L+S AG · Mangelsfeld 4 · D-97708 Bad Bocklet-Großenbrach

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e-mail: Labor@Labor-LS.de · Internet: www.Labor-LS.de
Akkreditiert nach ISO/IEC 17025 · Zertifiziert nach DIN EN ISO 14001

Durch das DAP
Deutsches Akkreditierungssystem Prüfwesen GmbH
akkreditiertes Prüflaboratorium.
Die Akkreditierung gilt für die in der Urkunde aufgeführten Verfahren.

Deutscher Akkreditierungs-Rat
DAP-PL-2542.00

Merck Biosciences AG
Attn. Dr. H. Märki
Weidenmattweg 4

CH -4448 Läufelfingen

L + S – Nr.: 06559654

Date: 28.06.04
Page 1 of 1

TEST REPORT on the Test for Abnormal Toxicity

Sample : Her2/neu (435-443)
Batch-No. : ProduktNr.: 05-59-0438
Designation :
Date of message: 04.06.2004 Start of examination: 21.06.2004
Arrival : 09.06.2004 End of examination : 28.06.2004

Performance :

- Method: according to USP 27/ Ph. Eur. 4th edition.
- Test solution: The content of one vial was dissolved with 3.5 ml aqua ad iniect.
- Test: 1st test
- Animals: 5 mice/2 guinea-pigs
- Application: i.p. injection
- Dose per mouse: 0.5 ml
- Dose per guinea-pigs: 1.0 ml
- Duration: 7 d

Species	Mice					Guinea-pigs	
Animal No.	1	2	3	4	5	1	2
Weight in g: beginn of test	17.6	18.4	18.5	17.9	17.5	266	274
Weight in g: end of test	18.9	19.9	19.7	19.5	19.2	282	291

Annotation: The content of one vial (2 mg) was dissolved with 3.5 ml water for injection. Dose per mouse 0.5 ml (pure). Dose per guinea pig 1.0 ml (1:2 with water for injection diluted).

Result:
All animals survived, no symptoms or body weight reduction were observed

The substance meets the requirements

Dr. Lothar Bomblies

i.V. Dr. Dorothee Jäger
(Head of Department)

DAMD17-01-1-0318

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Deutscher Akkreditierungs-Rat
DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - No.: 06612784

Date: July 12, 2004 / IT
Page :1 of 1

Test Report on the Test for Pyrogens

Sample : **HER 2/neu (435-443)**
Description : **Batch No.: AC 0522**
Reference : **Lieferscheinnr.: 02554**
Date of order : 06-08-2004
Sample receipt : 06-25-2004

Start of Examination : 06-29-2004
End of Examination : 06-29-2004

Procedure:

Realization: according to USP 27/ Ph. Eur. 4th edition.
Application: as described under "remark"
Injected volume: 1 ml/kg corresponding to a dose of: 0,03 mg/kg
Remark: The content of one Vial was dissolved with 35 ml of 0.9% sodium chloride solution.
Test: first Test

Measuring:

Animal No.	Temp. in °C before Injection 90'	60'	30'	0'	Injection	Temp. in °C after Injection 30'	60'	90'	120'	150'	180'
1	39,2	39,2	39,2	39,1		39,1	39,1	39,1	39,1	39,1	39,1
2	39,4	39,4	39,4	39,3		39,3	39,3	39,3	39,3	39,3	39,2
3	39,3	39,3	39,2	39,2		39,2	39,2	39,3	39,3	39,3	39,2

Evaluation:

Animal No.	Initial Temp. in °C (Mean of 30' and 0')	Maximum Temp. in °C (after Injection)	Difference in °C
1	39,15	39,1	0,00
2	39,35	39,3	0,00
3	39,20	39,3	0,10
		Sum:	0,10

Result/Conclusion:

The sample passes the test for pyrogens.

Instead of a sham test according to USP 27, a preliminary test according to Ph. Eur. 4th edition was done.

Dr. Lothar Bomblies

i. V. Dr. Jürgen Balles (Abteilungsleitung)

DAMD17-01-1-0318

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DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - Nr.: 06612784

Date: July 14, 2004 / UM
Page :1 of 1

Test Report on the Test for Sterility

Sample	: **HER 2/neu (435-443)**		
Description	: **Batch No.: AC 0522**		
Reference	: **Lieferscheinnr.: 02554**		
Date of order	: 06-08-2004	Start of Examination:	06-25-2004
Sample receipt	: 06-25-2004	End of Examination :	07-09-2004

Procedure:

- Number of samples tested: 20
- Membrane filtration according to Ph. Eur. 4th Edition/ USP 27
- Samples tested pooled
- Media used: Thioglycolat and CASO
- Test systems used: TTHA DV (Millipore)
- Incubation time: 14 days
- Special procedures: Dissolving and neutralization of the sample with peptone buffer.

Result / Conclusion

Sterile

The result complies with the requirements of the Test for Sterility

Remark: Growth control according to L+S SOP was performed.
The results comply with the requirements.

ppa. Dr. Frank Böttcher

i.V. Dr. Timo Krebsbach (Abteilungsleitung)

AMERICAN PEPTIDE

Synthesis and Purification Protocol for Her2/neu (435-443) C-S-242

Product Number APC: 322157
Lot Number APC: S05024A1
Sequence: Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu

The peptide was synthesized on Wang resin via Fmoc chemistry (see the attachment for details). Protecting groups used for amino acids are: t-Butyl group for Ser and Tyr; Trityl group for Asn and His. Fmoc protected amino acids were purchased from EMD Biosciences and Senn Chemicals USA. Reagents for coupling and cleavage were purchase from Aldrich. Solvents were purchased from Fish Scientific. The peptide chain was assembled on resin by repetitive removal of the Fmoc protecting group and coupling of protected amino acid. DIC and HOBt were used as coupling reagent and N-methylmorpholine was used as base. After removal of last Fmoc protecting group, resin was treated with TFA/TIS/H2O (95:3:2 v/v) cocktail for cleavage and removal of the side chain protecting groups. Crude peptide was precipitated from cold ether and collected by filtration. Purification of crude peptide was achieved via RP-HPLC using 47mm x 300mm column from Waters. Peptide was purified using triethyl phosphate buffer (buffer A) and acetonitrile (buffer B). A linear gradient of 5% to 35% buffer B in 60 minutes was used. Pooled fractions were desalted using acetic acid buffer. The peptide has been verified by MS analysis and amino acid analysis. The peptide purity was determined by analytical HPLC column (C18, 4.6 x 250mm) which was obtained from Supelco.

List of chemicals and solvents used in the synthesis
NMM (N-methylmorpholine)
DIC (Diisopropylcarbodiimide)
HOBt (1-Hydroxybenzotriazole)
Piperidine
TIS (Diisopropylsilane)
DMF (Dimethylformamide)
Diethylether
Trifluoroacetice acid
DCM (Dichloromethane)
Methanol

Attachment

Synthesis Procedure for Peptide

Product Number: 322157

AMERICAN PEPTIDE

Lot Number: S05024A1
Sequence: Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu

Step 1 (Resin swelling)

Fmoc-Leu-Wang resin was swelled in DCM for 30 minutes (10 ml/g resin)

Step 2 (Deprotection)

a. Add 20% piperidine/DMF solution (10 ml/g resin) to the resin.
b. Stir for 30 minutes (start timing when all the resin is free floating in the reaction vessel).
c. Drain.

Step 3 (Washing)

a. Wash the resin with DMF (10 ml/g resin) five times. Ninhydrin test: positive.

Step 4 (Coupling)

Fmoc-AA-OH:	3 equivalent relative to resin loading
HOBt:	3 equivalent relative to resin loading
DIC:	3 equivalent relative to resin loading
NMM:	6 equivalent relative to resin loading

Weigh Fmoc-AA-OH and HOBt into a plastic bottle. Dissolve the solids with DMF (5 ml/g resin). Add DIC to the mixture, followed by the addition of NMM . Add the mixture to the resin. Bubble (or stir) gently for 10 – 60 minutes until a negative ninhydrin test on a small sample of resin is obtained.

Step 5 (Washing)

Wash the resin with DMF (3 times).

Step 6

a. Repeat steps 2-5 until required peptide is assembled.

Step 7 (N-terminal Fmoc de-protection)

a. Repeat step 2 and go to step 8.

Step 8 (Washing and Drying)

a. After the final coupling, wash resin with DMF (3 times), MeOH (1 time), DCM (3 times) and MeOH (2 times).
b. Dry the resin under vacuum (water aspirator) for 2 hours and high vacuum (oil pump) for a minimum of 12 hours.

Cleavage

a. Place dry resin in a plastic bottle and add the cleavage cocktail. Shake the mixture at room temperature for 2.5 hours.
b. Remove the resin by filtration under reduced pressure. Wash the resin twice with TFA. Combine filtrates, and add an 8-10 fold volume of cold ether to get precipitate.
c. Crude peptide was isolated by filtration. Wash the crude peptide with cold ether (twice).

AMERICAN PEPTIDE

HER2/neu (435-443)
ILHNGAYSL

June 11, 2004

To: whom it may concern
From: American Peptide Company, Inc.

RE: Statement of Origin of Raw Materials

We, American Peptide Co, hereby confirmed that we purchased all the Fmoc protected L-amino acids used in the production of peptide **322157** (Lot Number: S05024A1) from EMD Bioscience, Inc and Senn Chemicals USA. According to our vendor's statement, all the L-amino acids are of synthetic or plant origin.

Baosheng Liu

Baosheng Liu, Ph.D
Vice President of R&D and Manufacturing
American Peptide Company, Inc.
Tel: 408-733-7604
Fax: 408-733-9057
e-mail: Baosheng@americanpeptide.com

DAMD17-01-1-0218 ®

CERTIFICATE OF ANALYSIS for CLINALFA® PRODUCTS

PRODUCT NAME: **HER 2/neu (883-899)**
PRODUCT NUMBER: **C- S-243** BATCH NUMBER: **AC 0523**
ACTIVE INGREDIENT NAME: H-Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe-OH (KVPIKWMALESILRRRF)
ACTIVE INGREDIENT FORMULA: C100 H167 N29 O21 S1 MOLECULAR WEIGHT: 2143.7

TESTS (Methods)	SPECIFICATIONS	RESULTS at RELEASE 6-Aug-04	RESULTS at RETEST
IDENTITY of Active Ingredient (coeleution, HPLC/UV)	Single Principal Peak	complies	
VIAL CONTENT of Active Ingredient (mean of N vials, HPLC/UV)	0.9 - 1.1 mg /N=6	1.0 mg /N=6	
UNIFORMITY of Vial Content (mean of N vials, HPLC/UV)	CV ≤ 5 % /N=6 (CV=Coefficient of Variation)	1.9 % /N=6	
ACTIVE INGREDIENT RELATED IMPURITIES (mean of N vials, HPLC/UV)	Total ≤ 5 % /N=6 Any ≤ 3 % /N=6	0.4 % /N=6 complies	
ACTIVE INGREDIENT PURITY (mean of N vials, HPLC/UV)	≥ 95 % /N=6	99.6 % /N=6	
STERILITY (thioglycollate & CASO, EP/USP membrane filtration method)	No evidence of microbial growth	complies	
PYROGENS (body temperature in rabbits, EP/USP method, i.v. dose: 0.03 mg /kg)	Summed response in 3 rabbits ≤ 1.15 °C	complies	

N. D. = NO DATA

NOTES:
Excipients: -
Soluble in 0.9 % NaCl
Vials sealed under N2.

Store at ≤ -18 °C, protect from light.
Estimated Expiry Date (Retest Date): 4-Feb-05

DATE: 6-Aug-04

A. Sauter, PhD
Director Quality Management

To be Used in Approved Clinical Trials Only!

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CERTIFICATE OF ANALYSIS for CLINALFA® ACTIVE INGREDIENTS

PRODUCT (API) NAME:	HER 2/neu (883-899)		
API NUMBER:	05-59-0439	API BATCH NUMMER: A 31076	MOL Wt.: 2143.7
CHEMICAL NAME: (AA SEQUENCE)	H-Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe-OH (KVPIKWMALESILRRRF)		FORMULA: C100 H167 N29 O21 S1

TESTS	SPECIFICATIONS	RESULTS
Appearance	white to off white, amorphous powder	white, amorphous powder
Mass Spectrum	ES-MS: monoisotopic, non-protonated mass = 2142.3 ± 1 Da (deduced from series of m/z signals)	2142.3 Da
Amino Acids	Ala: 1.0 Arg: 3.0 Glx: 1.0 Ile: 2.0 Leu: 2.0 Lys: 2.0 Met: 1.0 Phe: 1.0 Pro: 1.0 Ser: 1.0 Trp: 1.0 Val: 1.0 (standard values)	Ala: 1.1 Arg: 3.3 Glx: 1.1 Ile: 1.7 Leu: 2.0 Lys: 2.0 Met: 1.1 Phe: 1.0 Pro: 1.0 Ser: 1.0 Trp: *) Val: 1.0 (ADOPTED DATA)
Purity (HPLC/UV)	Impurities < 2 % Purity > 98 % (product related impurities)	99.7 %
Net Peptide Content	not specified (elementary N-analysis)	83.4 %
Water (GC)	not specified	5.2 %
Acetic Acid	not specified	5.4 %
Trifluoro Acetic Acid	< 1 %	**)1.1 %
Acetonitrile	< 400 ppm	<28 ppm
Isopropanol	< 400 ppm	<19 ppm
INFORMATION	**Store dry at ≤ -18 °C** N. D. = NO DATA *) Trp was completely destroyed during hydrolysis **) meets specification of 1% (not 1.0%) when rounded	

DATE: 21-Jun-04

A. Sauter, PhD
Director Quality Management

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DAMD17-01-1-0318

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Deutscher Akkreditierungs-Rat
DAP-PL-2542.00

L + S – Nr.: 06559664

Date: 28.06.04

TEST REPORT on the Test for Abnormal Toxicity

Sample : Her2/neu (883-899)
Batch-No. : ProduktNr.: 05-59-0439
Designation :
Date of message: 04.06.2004 Start of examination: 21.06.2004
Arrival : 09.06.2004 End of examination : 28.06.2004

Performance :

- Method: according to USP 27/ Ph. Eur. 4th edition.
- Test solution: The content of one vial was dissolved with 3.5 ml aqua ad iniect.
- Test: 1st test
- Animals: 5 mice/2 guinea-pigs
- Application: i.p. injection
- Dose per mouse: 0.5 ml
- Dose per guinea-pigs: 1.0 ml
- Duration: 7 d

Species	Mice					Guinea-pigs	
Animal No.	1	2	3	4	5	1	2
Weight in g: beginn of test	17.9	18.5	17.6	18.1	18.5	261	274
Weight in g: end of test	19.1	20.2	19.3	19.5	20.3	270	282

Annotation: The content of one vial (2 mg) was dissolved with 3.5 ml water for injection. Dose per mouse 0.5 ml (pure). Dose per guinea pig 1.0 ml (1:2 with water for injection diluted).

Result:
All animals survived, no symptoms or body weight reduction were observed

The substance meets the requirements

Dr. Lothar Bomblies

i.V. Dr. Dorothee Jäger
(Head of Department)

DAMD17-01-1-0318

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DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - Nr.: 07700534

Date: July 29, 2004 / UM

Test Report on the Test for Sterility

Sample	: **HER 2/neu (883-899)**		
Description	: **Lot: AC 0523, BAG-Ch.-B.: 429602**		
Reference	:		
Date of order	: 07-13-2004	Start of Examination:	07-15-2004
Sample receipt	: 07-15-2004	End of Examination :	07-29-2004

Procedure:

- Number of samples tested: 20
- Membrane filtration according to Ph. Eur., 4th Edition/USP27
- Samples tested pooled
- Media used: Thioglycolat and CASO
- Test systems used: TTHA DV (Millipore)
- Incubation time: 14 days
- Special procedures: Dissolving and neutralization of the sample with peptone buffer.

Result / Conclusion

Sterile

The result complies with the requirements of the Test for Sterility

ppa. Dr. Frank Böttcher

i.V. Dr. Timo Krebsbach (Abteilungsleitung)

DAMD17-01-1-0318

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DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
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L + S - No.: 07700534

Date: July 29, 2004 / UM
Page :1 of 1

Test Report on the Test for Pyrogens

Sample : **HER 2/neu (883-899)**
Description : **Lot: AC 0523, BAG-Ch.-B.: 429602**
Reference :
Date of order : 07-13-2004
Sample receipt : 07-15-2004

Start of Examination : 07-22-2004
End of Examination : 07-22-2004

Procedure:

Realization: according to USP 27/ Ph. Eur. 4th edition.
Application: as described under "remark"
Injected volume: 1 ml/kg corresponding to a dose of: 0.03 mg/kg
Remark: The content of one Vial was dissolved with 35 ml of 0.9% sodium chloride solution.
Test: first Test

Measuring:

Animal No.	Temp. in °C before Injection 90'	60'	30'	0'	Injection	Temp. in °C after Injection 30'	60'	90'	120'	150'	180'
1	39,2	39,3	39,2	39,2		39,2	39,2	39,2	39,2	39,1	39,1
2	39,3	39,3	39,2	39,2		39,1	39,1	39,1	39,1	39,1	39,0
3	39,6	39,6	39,6	39,5		39,5	39,5	39,4	39,4	39,3	39,2

Evaluation:

Animal No.	Initial Temp. in °C (Mean of 30' and 0')	Maximum Temp. in °C (after Injection)	Difference in °C
1	39,20	39,2	0,00
2	39,20	39,1	0,00
3	39,55	39,5	0,00
		Sum:	0,00

Result/Conclusion:

The sample passes the test for pyrogens.

Instead of a sham test according to USP 27, a preliminary test according to Ph. Eur. 4th edition was done.

Dr. Lothar Bomblies

i. V. Dr. Dorothee Jäger (Abteilungsleitung)

AMERICAN PEPTIDE

Synthesis and Purification Protocol for Her2/neu (883-889) C-S-243

Product Number APC: 311901
Lot Number APC: S05082A1
Sequence: Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe

The peptide was synthesized on Wang resin via Fmoc chemistry (see the attachment for details). Protecting groups used for amino acids are: t-Butyl group for Glu and Ser; Pbf for Arg; Boc for Lys and Trp. Fmoc protected amino acids were purchased from EMD Biosciences and Senn Chemicals USA. Reagents for coupling and cleavage were purchase from Aldrich. Solvents were purchased from Fish Scientific. The peptide chain was assembled on resin by repetitive removal of the Fmoc protecting group and coupling of protected amino acid. HBTU and HOBt were used as coupling reagent and N-methylmorpholine was used as base. After removal of last Fmoc protecting group, resin was treated with TFA/Thioanisole/Phenol/H_2O/EDT (87.5:5:2.5:2.5:2.5v/v) cocktail for cleavage and removal of the side chain protecting groups. Crude peptide was precipitated from cold ether and collected by filtration. Purification of crude peptide was achieved via RP-HPLC using 47mm x 300mm column from Waters. Peptide was first purified using triethyl phosphate buffer (buffer A) and acetonitrile (buffer B). A linear gradient of 18% to 50% buffer B in 60 minutes was used. Pooled fractions were then purified using TFA buffer (Buffer A: 0.1% TFA in H_2O, Buffer B: acetonitrile). A linear gradient of 15% to 55% buffer B in 60 minutes was used. Pooled fractions were desalted using acetic acid buffer. The peptide has been verified by MS analysis and amino acid analysis. The peptide purity was determined by analytical HPLC column (C18, 4.6 x 250mm) which was obtained from Supelco.

List of chemicals and solvents used in the synthesis

NMM (N-methylmorpholine)
HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate)
HOBt (1-Hydroxybenzotriazole)
Piperidine
Thioanisole
Phenol
EDT (1,2-Ethanedithiol)
DMF (Dimethylformamide)
Diethylether
Trifluoroacetice acid
DCM (Dichloromethane)
Methanol

AMERICAN PEPTIDE

Attachment

Synthesis Procedure for Peptide

Product Number: 311901
Lot Number: S05082A1
Sequence: Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe

Step 1 (Resin swelling)

Fmoc-Phe-Wang resin was swelled in DCM for 30 minutes (10 ml/g resin)

Step 2 (Deprotection)

a. Add 20% piperidine/DMF solution (10 ml/g resin) to the resin.
b. Stir for 30 minutes (start timing when all the resin is free floating in the reaction vessel).
c. Drain.

Step 3 (Washing)

a. Wash the resin with DMF (10 ml/g resin) five times. Ninhydrin test: positive.

Step 4 (Coupling)

Fmoc-AA-OH:	3 equivalent relative to resin loading
HOBt:	3 equivalent relative to resin loading
HBTU:	3 equivalent relative to resin loading
NMM:	6 equivalent relative to resin loading

Weigh Fmoc-AA-OH and HOBt into a plastic bottle. Dissolve the solids with DMF (5 ml/g resin). Add HBTU to the mixture, followed by the addition of NMM . Add the mixture to the resin. Bubble (or stir) gently for 10 – 60 minutes until a negative ninhydrin test on a small sample of resin is obtained.

Step 5 (Washing)

Wash the resin with DMF (3 times).

Step 6

a. Repeat steps 2-5 until required peptide is assembled.

Step 7 (N-terminal Fmoc de-protection)

a. Repeat step 2 and go to step 8.

Step 8 (Washing and Drying)

a. After the final coupling, wash resin with DMF (3 times), MeOH (1 time), DCM (3 times) and MeOH (2 times).
b. Dry the resin under vacuum (water aspirator) for 2 hours and high vacuum (oil pump) for a minimum of 12 hours.

Cleavage

a. Place dry resin in a plastic bottle and add the cleavage cocktail. Shake the mixture at room temperature for 2.5 hours.
b. Remove the resin by filtration under reduced pressure. Wash the resin twice with TFA. Combine filtrates, and add an 8-10 fold volume of cold ether to get precipitate.
c. Crude peptide was isolated by filtration. Wash the crude peptide with cold ether (twice).

AMERICAN PEPTIDE

HER2/neu (883-899)
KVPIKWMALESILRRF

June 11, 2004

To: whom it may concern
From: American Peptide Company, Inc.

RE: Statement of Origin of Raw Materials

We, American Peptide Co, hereby confirmed that we purchased all the Fmoc protected L-amino acids used in the production of peptide **311901** (Lot Number: S05082A1) from EMD Bioscience, Inc and Senn Chemicals USA. According to our vendor's statement, all the L-amino acids are of synthetic or plant origin.

Baosheng Liu

Baosheng Liu, Ph.D
Vice President of R&D and Manufacturing
American Peptide Company, Inc.
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e-mail: Baosheng@americanpeptide.com

DAMD17-01

CERTIFICATE OF ANALYSIS for CLINALFA PRODUCTS

PRODUCT NAME: **MUC 1 (578-586)**
PRODUCT NUMBER: **C- S-251** BATCH NUMBER: **AC 0541**

ACTIVE INGREDIENT NAME: Ser-Thr-Ala-Pro-Pro-Val-His-Asn-Val (STAPPVHNV)

ACTIVE INGREDIENT FORMULA: C40 H64 N12 013 MOLECULAR WEIGHT: 921.0

TESTS (Methods)	SPECIFICATIONS	RESULTS at RELEASE 10-Nov-04	RESULTS at RETEST
IDENTITY of Active Ingredient (coeleution, HPLC/UV)	Single Principal Peak	complies	
VIAL CONTENT of Active Ingredient (mean§) of N vials, HPLC/UV)	0.9 - 1.1 mg /N=6	1.0 mg /N=6	
UNIFORMITY of Vial Content (mean of N vials, HPLC/UV)	CV ≤ 5 % /N=6 (CV=Coefficient of Variation)	4.3 % /N=6	
ACTIVE INGREDIENT RELATED IMPURITIES (mean of N vials, HPLC/UV)	Total ≤ 5 % /N=6 Any ≤ 3 % /N=6	0.1 % /N=6 complies	
ACTIVE INGREDIENT PURITY (mean of N vials, HPLC/UV)	≥ 95 % /N=6	99.9 % /N=6	
STERILITY (membrane filtration method, thioglycollate & CASO media)	No evidence of microbial growth	complies	
PYROGENS (body temperature in rabbits, i.v. dose: 0.03 mg /kg)	Summed response in 3 rabbits ≤ 1.15 °C	complies	

§) ROUNDING INTERVAL: 0.2 mg N. T. = NOT TESTED

NOTES:

Excipients: not used
Soluble in 0.9 % NaCl
Vials sealed under N2. Store at ≤ -18 °C, protect from light.

TO BE USED UNTIL (DATE OF RETEST): 11-May-05

DATE: 11-Nov-04

A. Sauter, PhD
Director Quality Management
CLINALFA® Product Line
Phone +41 62 285 25 50
Fax +41 62 285 25 20
email clinalfa@merckbiosciences.ch
www.clinalfa.com

TO BE USED IN APPROVED CLINICAL TRIALS ONLY!

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TSE / BSE CERTIFICATE for CLINALFA PRODUCTS

PRODUCT NAME: **MUC 1 (578-586)**

PRODUCT NUMBER: **C- S-251** BATCH NUMBER: **AC 0541**

ACTIVE INGREDIENT NAME: Ser-Thr-Ala-Pro-Pro-Val-His-Asn-Val (STAPPVHNV)

ACTIVE INGREDIENT FORMULA: C40 H64 N12 013 MOLECULAR WEIGHT: 921.0

We herewith certify that the Batch of the Product mentioned above has been manufactured form starting materials (active and inactive ingredients, additives, reagents) of category IV, and using processes that do not represent any risk of contamination with pathogens of transmissible spongiform encephalopathies (TSE/BSE).

The Product is in full compliance with EMEA /410/01 rev. 1, respectively the European Council Directive 75/318/ECC.

DATE: 11-Nov-04

A. Sauter, PhD
Director Quality Management

To be Used in Approved Clinical Trials Only!

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CERTIFICATE OF ANALYSIS for CLINALFA ACTIVE INGREDIENTS

PRODUCT (API) NAME: MUC 1, aa 578-586		
API NUMBER: 05-59-0450	API BATCH NUMMER: A 31623	MOL Wt.: 921.0
CHEMICAL NAME: (AA SEQUENCE) Ser-Thr-Ala-Pro-Pro-Val-His-Asn-Val (STAPPVHNV)		FORMULA: C40 H64 N12 013

TESTS	SPECIFICATIONS	RESULTS
Appearance	white to off white, amorphous powder	white, amorphous powder
Mass Spectrum	ES-MS: monoisotopic, non-protonated mass = 920.5 ± 1 Da (deduced from series of m/z signals)	920.5 Da
Amino Acids	Ala: 1.0 Asx: 1.0 His: 1.0 Pro: 2.0 Ser: 1.0 Thr: 1.0 Val: 2.0 (standard values)	Ala: 1.0 Asx: 1.0 His: 1.0 Pro: 2.0 Ser: 0.9 Thr: 1.0 Val: 2.0 (ADOPTED DATA)
Purity (API related impurities)	Impurities < 1 % Purity > 99 % (HPLC/UV)	100.0 %
Net Peptide Content	not specified (elementary N-analysis)	85.5 %
Water	not specified (gaschromatography)	8.4 %
Acetic Acid	not specified (gaschromatography)	3.0 %
Trifluoro Acetic Acid	< 1 %	< 0.1 % (ADOPTED DATA)
Acetonitrile	< 400 ppm (gaschromatography)	< 64 ppm
Triethylamine	< 0.8 % (gaschromatography)	0.2 %
Phosphate	not specified (elementary P-analysis)	2.8 %
INFORMATION	N. D. = NO DATA	**Store dry at ≤ -18 °C**

DATE: 8-Oct-04

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DAMD17-01-1-0318

Labor L+S AG · Mangelsfeld 4 · D-97708 Bad Bocklet-Großenbrach

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e-mail: Labor@Labor-LS.de · Internet: www.Labor-LS.de
Akkreditiert nach ISO/IEC 17025 · Zertifiziert nach DIN EN ISO 14001

Durch das DAP
Deutsches Akkreditierungssystem Prüfwesen GmbH
akkreditiertes Prüflaboratorium.
Die Akkreditierung gilt für die in der Urkunde aufgeführten Verfahren.

Deutscher Akkreditierungs-Rat
DAP-PL-2542.00

Merck Biosciences AG
z.Hd. Herrn A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - Nr.: 10062824

Date: November 8, 2004 / UM
Page :1 of 1

Test Report on the Test for Sterility

Sample	: **MUC-1 (578-586)**
Description	: **LOT Nr.: AC 0541, BAG Ch.B.: 443605**
Reference	: **Fa. BAG, Lich**
Date of order	: 10-22-2004
Sample receipt	: 10-25-2004
Start of Examination:	10-25-2004
End of Examination :	11-08-2004

Procedure:

- Number of samples tested: 20
- Membrane filtration according to Ph. Eur., 4th Edition/ USP 27
- Samples tested pooled
- Media used: Thioglycolat and CASO
- Test systems used: TTHA DV (Millipore)
- Incubation time: 14 days
- Special procedures: Dissolving and neutralization of the sample with peptone buffer.

Result / Conclusion

Sterile

The result complies with the requirements of the Test for Sterility

ppa. Dr. Frank Böttcher

i.V. Dr. Timo Krebsbach (Abteilungsleitung)

DAMD17-01-1-0318

Labor L+S AG · Mangelsfeld 4 · D-97708 Bad Bocklet-Großenbrach

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Weidenmattweg 4

CH -4448 Läufelfingen

L + S - No.: 10062824

Date: November 8, 2004 / UM
Page :1 of 1

Test Report on the Test for Pyrogens

Sample : **MUC-1 (578-586)**
Description : **LOT Nr.: AC 0541, BAG Ch.B.: 443605**
Reference : **Fa. BAG, Lich**
Date of order : 10-22-2004
Sample receipt : 10-25-2004

Start of Examination : 10-26-2004
End of Examination : 10-26-2004

Procedure:

Realization: according to USP 27/ Ph. Eur. 4th. edition.
Application: as described under "remark"
Injected volume: 1 ml/kg corresponding to a dose of: 0.03 mg/kg
Remark: The content of one Vial was dissolved with 35 ml of 0.9% sodium chloride solution.
Test: first Test

Measuring:

Animal No.	Temp. in °C before Injection 90'	60'	30'	0'	Injection	Temp. in °C after Injection 30'	60'	90'	120'	150'	180'
1	39,3	39,2	39,1	39,1		39,1	39,1	39,1	39,1	39,1	39,1
2	39,0	39,0	38,9	38,9		38,9	38,8	38,8	38,8	38,8	38,8
3	39,0	39,0	39,0	39,0		39,1	39,1	39,1	39,1	39,1	39,1

Evaluation:

Animal No.	Initial Temp. in °C (Mean of 30' and 0')	Maximum Temp. in °C (after Injection)	Difference in °C
1	39,10	39,1	0,00
2	38,90	38,9	0,00
3	39,00	39,1	0,10
		Sum:	0,10

Result/Conclusion:

The sample passes the test for pyrogens.

Instead of a sham test according to USP 27, a preliminary test according to Ph. Eur. 4th edition was done.

Dr. Lothar Bomblies

i. V. Dr. Jürgen Balles (Abteilungsleitung)

DAMD17-01-1-0318

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Durch das DAP
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akkreditiertes Prüflaboratorium.
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Deutscher Akkreditierungs-Rat
DAP-PL-2542.00

L + S – Nr.: 10002194

Date: 27.10.04
Page 1 of 1

TEST REPORT on the Test for Abnormal Toxicity

Sample	: Muc 1 (578-586)	
Description	: 05-59-0450, A31623, Produkt-Nr. C-S-251 / AC0541	
Reference	:	
Date of order	: 06.10.2004	Start of examination: 20.10.2004
Sample receipt	: 08.10.2004	End of examination : 27.10.2004

Performance :

- Method: according to USP 27 / Ph.Eur. 4th edition
- Test: 1st test
- Animals: 5 mice/2 guinea-pigs
- Nominal weight: 17 to 22/250 to 350 g
- Dose per animal: 0.5/1.0 ml
- Application: i. p.
- Duration: 7 d
- Remark: The content of one vial (7 mg) was dissolved with 3.5 ml water for injection. Dose per mouse 0.5 ml (pure). Dose per guinea pig 1.0 ml (1:2 with water for injection diluted).

Species	Mice					Guinea-pigs	
Animal No.	1	2	3	4	5	1	2
Weight in g: begin of test	20.9	20.2	20.5	19.2	19.5	303	314
Weight in g: end of test	22.6	21.5	22.3	20.9	21.1	314	329

Result:

All animals survived, no symptoms were observed

The substance meets the test

Dr. Lothar Bomblies

i.V. Dr. Jürgen Balles
(Head of Department)

Synthesis and Purification Protocol for Peptide

Product Number: **309235**
Lot Number: **S09098A1**
Sequence: **Ser-Thr-Ala-Pro-Pro-Val-His-Asn-Val**

The peptide was synthesized on Wang resin via Fmoc chemistry (see the attachment for details). Protecting groups used for amino acids are: t-Butyl group for Ser and Thr; Trt for His and Asn. Fmoc protected amino acids were purchased from EMD Biosciences and Senn Chemicals USA. Reagents for coupling and cleavage were purchase from Aldrich. Solvents were purchased from Fish Scientific. The peptide chain was assembled on resin by repetitive removal of the Fmoc protecting group and coupling of protected amino acid. HBTU and HOBt were used as coupling reagent and N-methylmorpholine was used as base. After removal of last Fmoc protecting group, resin was treated with TFA/TIS/H2O (95:3:2 v/v) cocktail for cleavage and removal of the side chain protecting groups. Crude peptide was precipitated from cold ether and collected by filtration. Purification of crude peptide was achieved via RP-HPLC using 47mm x 300mm column from Waters. Peptide was purified using combination of 1% triethylammonium phosphate buffer (prepared by mixing equal volume of triethylamine and phosphoric acid in water) and acetonitrile. A linear gradient of 10% to 40% acetonitrile in 60 minutes was used. Pooled fractions were desalted using 0.5% acetic acid buffer and acetonitrile on reversed phase HPLC column. Pooled fractions were lyophilized. The peptide has been verified by MS analysis and amino acid analysis. The peptide purity was determined by analytical HPLC column (C18, 4.6 x 250mm) which was obtained from Supelco.

The last solvent mixture that the peptide was in contact with is 0.5% acetic acid and 30% acetonitrile in water.

Ala	A28366	EMD Biosciences, Inc
Asn	9550	Senn Chemicals USA
His	A30693	EMD Biosciences, Inc
Pro	A29140	EMD Biosciences, Inc
Ser	A30145	EMD Biosciences, Inc
Thr	A29257	EMD Biosciences, Inc
Val	A29139	EMD Biosciences, Inc

List of chemicals and solvents used in the synthesis
NMM (N-methylmorpholine)
HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate)
HOBt (1-Hydroxybenzotriazole)
Piperidine
TIS (Diisopropylsilane)
DMF (Dimethylformamide)
Diethylether
Trifluoroacetice acid
DCM (Dichloromethane)
Methanol

ASSISTANCE AGREEMENT

DAMD17-01-1-0318

AWARD TYPE: [✓] GRANT (31 USC 6304) [ ] COOPERATIVE AGREEMENT (31 USC 6305) [ ] OTHER TRANSACTION (10 USC 2371)			
AWARD NO: DAMD17-01-1-0318 Modification P00001	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT $1,901,322.00	Page 1 of 1 Pamela Fisher 301-619-2805 301-619-4084 (FAX)

PROJECT TITLE: "Immunotherapeutic Strategies in Breast Cancer: Preclinical and Clinical"

CFDA 12.420

PERFORMANCE PERIOD: 15 August 2001 - 14 September 2007 (Research ends 14 August 2007)	**PRINCIPAL INVESTIGATOR:** Sandra J. Gendler, Ph.D
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-R 820 Chandler St. Fort Detrick Maryland 21702-5014	**PAYMENTS WILL BE MADE BY:** EFT:T Army Vendor Pay DFAS-SA/FPA (888)478-5636 500 McCullough Avenue San Antonio, TX 78215
DUNS No: 15366-5211 **TIN No:**	*(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)*
AWARDED TO: Mayo Clnic Scottsdale 13400 E. Shea Blvd Scottsdale, AZ 85259	**REMIT PAYMENT TO:** Mayo Clnic Scottsdale 13400 E. Shea Blvd Scottsdale, AZ 85259

ACCOUNTING AND APPROPRIATION DATA: N/A

SCOPE OF WORK:

BREAST CANCER RESEARCH PROGRAM

CLINICAL TRANSLATIONAL AWARD

1. In accordance with the Assistance Agreement, Para. 4.c., and the recipient's letter notification dated 20 January 2005, which is incorporated herein by reference, the Assistant Agreement "performance period" end date is extended by 24 months. Funding is unchanged. Details are as follows:

   FROM: 15 August 2001 through 14 September 2005 (Research ends 14 August 2005)

   TO: 15 August 2001 through 14 September 2007 (Research ends 14 August 2007)

2. There are no other changes to the grant agreement.

TOTAL AMOUNT OF AWARD: $1,901,322.00

RECIPIENT		GRANTS OFFICER	
ACCEPTED BY: No signature required. Reference recipient's request dated January 20, 2005, SIGNATURE		**UNITED STATES OF AMERICA** SIGNATURE	
NAME AND TITLE	DATE 2/24/05	NAME AND TITLE Patricia A. Evans GRANTS OFFICER	DATE 4 Jan 2005

USAMRAA FORM 60-R, Feb 00