AD_____

Award Number: DAMD17-96-1-6322

TITLE: Immunity to HER-1/neu Protein

PRINCIPAL INVESTIGATOR: Mary L. Disis, M.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, Washington 98105-6613

REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (23 Sep 96 - 22 Sep 00)

4. TITLE AND SUBTITLE
Immunity to HER-1/neu Protein

5. FUNDING NUMBERS
DAMD17-96-1-6322

6. AUTHOR(S)
Mary L. Disis, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
Seattle, Washington 98105-6613

E-MAIL:
ndisis@u.washington.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

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

The HER-2/neu (HER2) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. HER2 over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. Preliminary studies prior to the grant discovered that some patients with breast cancer have existent CD4+ helper T cell immunity and antibody-mediated immunity to HER2. HER2 is a self protein. Therefore, before our studies it had been assumed that patients would be immunologically tolerant to HER2 and that immunity could not be generated. Our prior studies demonstrating that immunity is already present in some patients with breast cancer implied that immunity to HER2 is induced in some individuals by virtue of the presence of growing cancer expressing the antigen and gives credence to the concept that HER2-specific immunity can potentially be used in therapy without destroying normal tissue. The current grant is exploring issues important for developing HER2 specific vaccines and T cell therapy.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

INTRODUCTION:

The HER2 (HER2) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. HER2 over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. HER2 may also be related to cancer formation, with overexpression being detectable in 50-60% of ductal carcinomas *in situ* (DCIS). The overall goal for the proposal was to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. This final report demonstrates that both these aims have been accomplished. Work that has been accomplished, funded by this proposal, has led to the development of a HER2 vaccine trial funded by the NCI as well as a Phase I study of adoptive T cell therapy in advanced stage breast cancer recently funded by the NCI. This progress report details not only the progress on the original aims defined, but also new studies undertaken in line with the original grant goals. Along with two novel therapeutic approaches that have been translated into patients with breast cancer, work completed here has led to studies developing serologic methods for cancer diagnostics.

Specific Aim #1 is examining Ab immunity to HER2. Preliminary data showed that Ab immunity to HER2 could be detected in the sera of some patients with breast cancer. Studies were proposed to determine the frequency of Ab immunity, the relative frequency of functional Ab and to determine whether responses to HER2 are beneficial or detrimental. Additional studies were proposed to determine whether immunity to HER2 can serve as a marker for early cancer and/or whether changes in level represent a marker for relapse.

Specific Aim # 2 is examining CD4+ T cell immunity to HER2. Preliminary data showed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy. Studies proposed have demonstrated that HER2 specific CD4+ T cells can be detected and expanded from the peripheral blood of patients with breast cancer. In addition, an animal model has been developed to determine the role of CD4+ T cell immunity in mediating an anti-tumor response.

Specific Aim # 3 is examining CD8+ CTL immunity to HER2. Preliminary data for the grant showed that CD8+ CTL could be primed to HER2 peptides *in vitro* and that primed peptide-specific CTL can lyse HER2-positive cancer cells. However, the systems employed are extremely fastidious. *In vitro* priming was to be developed and used to identify the immunogenic epitopes of HER2. Additional studies were proposed to determine whether patients with breast cancer have existent CTL immunity to HER2, as had previously been described for patients with ovarian cancer. For patients with CTL immunity and HER2-positive cancers, studies were proposed to determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. Finally, studies were proposed to determine whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor and can be expanded *in vitro* to the extent needed for adoptive therapy.

Tasks defined under each aim have been completed as of this final progress report. In addition, several new areas of study have been launched based on data derived from these studies. Preliminary data developed during this granting period has been responsible for several additional projects and the following successful grant applications:

1. R01, *Phase I Trial of a Peptide Vaccine against EGFRvIII*, 1999-2002, NCI
2. R01, *Novel Vaccine Targets for Early Stage Breast Cancer*, 2000-2003, NCI
3. R01, *HER2 Specific T Cell Infusions Following HER2 Peptide Based Vaccination for the Treatment of HER2 Overexpressing Malignancy*, 2000-2005

In addition, studies described in this progress report and previous reports have led to the following translational clinical trials in patients with breast cancer:

1. A Phase I Study of Infusion of a HER-2/neu Specific T Cells in Patients with Advanced Stage HER-2/neu Expressing Cancers who Have Received a HER-2/neu Peptide Based Vaccine
2. A Phase I study of an EGFRvIII peptide based vaccine in patients with EGFRvIII expressing cancers
3. A Phase I Vaccine Trial of HER-2/neu Intracellular Domain (HER2 ICD) Protein and GM-CSF as Adjuvant in Patients with HER2-Overexpressing Breast or Ovarian Cancer

BODY:

The BODY of the Progress Report will be organized according to the STATEMENT OF WORK in the original grant proposal.

Specific Aim #1: To examine Ab immunity to HER2.

Task 1: Months 1-48 To determine the frequency of Ab, the biologic function of HER2 specific Ab, Ab correlation with HER2 overexpression and Ab correlation with circulating ECD. Previous progress reports have defined the incidence of HER2 antibody responses in breast, colon, and prostate cancer and demonstrated that the presence of HER2 antibodies correlates with protein expression in the tumor. In general, the overall incidence of HER2 antibodies in patients with HER2 overexpressing malignancies is about 20%. Previous progress reports (1999) demonstrated that there was no significant correlation between the presence and absence of circulating ECD protein and antibody response. Thus, the questions posed in Aim 1, Task 1 have been answered. The final focus of the funding period concentrated on developing antibody assays as a tool for breast cancer diagnostics, that is developing tests to standards that would allow large scale analysis of samples.

Most assays for the evaluation of serum tumor markers involve the direct measurement of a candidate protein in serum. Significant levels of the protein, however, must be circulating to allow detection of cancer. High concentrations of circulating protein generally require a bulky tumor mass; therefore, assays dependent on measuring shed tumor proteins directly in serum generally reflect advanced stage disease. Measurement and quantitation of antibody responses to infectious disease antigens have been used as a diagnostic and screening tool for decades. The detection of antibodies as a surrogate measure of infection by a particular virus, for instance, indicates exposure to that virus. The appearance of antibody or increase in antibody titer may serve as a measure or indicator of active infection. In fact, antibody immunity to an antigen can be detected even when the antigen itself is present only at very low levels. Direct antigen recognition by B cells results in a clonal amplification of antigen specific cells each producing antibody. So, in this fashion, the antibody response is an indicator of exposure to an infectious agent. If tumors were immunogenic and antibody responses were mounted to clinically relevant tumor related proteins, measurement of an antibody response to cancer may be a more sensitive diagnostic tool than direct measurement of shed tumor proteins.

Three common oncogene encoded proteins associated with breast cancer include, the growth factor receptor, HER2 (30% of cases), the tumor suppresser p53 (50% of cases), and the transcription factor, Myc (20% of cases). Moreover, evidence is accumulating that patients with various malignancies mount antibody responses to these oncogenic proteins. We have developed methods for the detection of HER2 and p53 proteins that approach CLIA standard in terms of their reproducibility (Table 1). Stored sera from over 100 cancer patients and 300 volunteer blood donors were used for assay validation.

Table 1. Development of antibody assays for clinical use.

PARAMETERS EVALUATED	RESEARCH ASSAYS		CLIA-BASED STANDARD
	HER2	P53	
Accuracy	12%	10%	<10%
Precision			
Intra assay	9%	12%	<10%
Inter assay	20%	15%	<10%
Specificity	77%	100%	>80%
Sensitivity	89%	93%	>90%
Linearity	r=0.98	r=0.95	r=0.95

Last years' report demonstrated that we have begun to screen for new antigens in breast cancer using the technique SEREX. We have identified 5 novel antigens in breast cancer, which have not been defined previously. Current studies are focused on developing high throughput methodologies of defining the incidence of immunogenicity of these novel antigens in breast cancer using an ELISA based technique. We have developed a screening method for the evaluation of potential novel tumor antigens that does not require the purification of

recombinant protein, but rather the construction of a mammalian expression vector encoding the DNA of interest with a polyhistidine tag. The full-length cDNA of the candidate antigen is cloned into the mammalian expression vector pcDNA3.1/His (Invitrogen). This vector has several attractive features: (1) expression of the inserted gene is driven by the CMV promoter and enhancer, which typically yields high-level expression in mammalian cells; (2) neomycin resistance gene for selecting transformants; (3) a multi-cloning site is present in three different reading frames to accommodate different inserts, and (4) the presence of the polyhistidine tag at the N-terminus of the recombinant protein to ensure its expression. The histidine tag can be detected with a monoclonal antibody specific for polyhistidine (Santa Cruz Biotechnology), which is used to bind recombinant proteins in an ELISA.

Once the candidate antigen gene is cloned into pcDNA3.1/His in the appropriate reading frame, the vector is used to transiently transfect a Chinese hamster ovary (CHO) cell line to produce recombinant protein. Using the pcDNA3.1 vector and Effectine transfection reagent (Qiagen), we have achieved good transfection efficiencies and have established stable protein-expressing transformants (data not shown). Expression of the recombinant protein of the predicted molecular weight is verified by Western blot using an anti-polyhistidine antibody (Santa Cruz Biotechnology). For evaluation of antibody responses, ELISA plates are coated with anti-polyhistidine antibody followed by the addition of lysates from the transfected CHO cells. Lysates from vector-transfected CHO cells are used in some wells to serve as a negative control. The polyhistidine-tagged recombinant protein binds to the anti-polyhistidine antibody and becomes immobilized on the plate. Serum samples from cancer patients and volunteer donor controls are then added to the wells, and any serum antibodies specific for the candidate protein will be detected after addition of enzyme-conjugated secondary antibodies to human IgG and IgA based on published methods.

Thus, the ability to develop assays that meet standard for wide scale screening of samples and the identification of a panel of breast cancer tumor antigens may allow the development of a multi-antigen screening test that may aid in the detection of breast cancer as well as identify novel antigens that may be useful in the development of multi-antigen breast cancer vaccines.

Task 2: Months 1-24 To examine biologic function of Ab binding to the ECD. Previous years' reports have demonstrated that (1) antibodies directed to the HER2 ECD can be found in the sera of patients and (2) ECD proteins can be constructed to immunize animals to generate ECD specific antibodies. Currently, ECD proteins based vaccines, based on previous data, are under construction for use in Phase I clinical trials in humans. However, protein based vaccines may be difficult to standardize from lot to lot and the uptake and processing of self-proteins by APCs *in vivo* is not well defined.

We have begun to evaluate the use of nucleic acid based vaccines encoding co-stimulatory molecules to specifically elicit ECD antibody responses. In collaboration with K.E. and I. Hellstrom, Seattle, WA, DNA vectors encoding HER antigen and co-stimulatory molecules were constructed. The control plasmid, pLNCX contains a CMV promoter. pLNC-4-1BBL was constructed by removing the murine 4-1BBL from pLXSHD by SfiI and EcoRI and cloning it into Hpa I-cut pLNCX vector. Murine CD80 was obtained by RT-PCR and cloned into pLNCX to generate pLNC -CD80. PLNC-CD80 was constructed by digesting CD80 PCR product with Cla I and Hind III and cloning it into a Cla I-Hind III-cut pLNCX vector. Murine CD86 was obtained by retrotranscription and PCR amplification of a 5 day murine ConA blast. pLNC-CD86 was constructed by digesting CD86 PCR product with Cla I and cloning it into a HpaI-cut pLNCX vector. pLNC-rat-neu was constructed by removing rat neu from pSV2 with Hind III and AfI III and cloning it into Hpa I-cut pLNCX. Plasmid DNA for vaccination was prepared with Qiagen Plasmid Maxi Kit (Qiagen Inc., Valencia, CA). Mice transgenic for rat neu develop cancers mediated by rat neu protein overexpression. Rat neu is a self-protein in these animals similar to HER2, a self-protein in humans. DNA immunization with vectors encoding co-stimulatory models could circumvent tolerance and generate rat neu specific antibody immunity in these animals. Neu-tg mice were immunized with vectors containing rat neu and various co-stimulatory molecules constructed as described above. After immunization, immune spleens were harvested, minced, and filtered through cell strainer. The relative number of B cells was increased in the group immunized with a combination of pLNC-Rat Neu+pLNC-4-1BB ligand and pLNC-Rat Neu+ pLNC-4-1BB ligand+pLNC-CD86 (Figure 1).

Figure 1. Mice immunized with HER2 vaccines encoding 4-1BBl +/- CD86 develop an increase in splenic B cells. Neu-tg mice were immunized with DNA vaccines encoding HER2 and various co-stimulatory molecules. The ratio of T-lymphocytes and B-lymphocytes was examined by staining with FITC anti-mouse CD3e clone 145-2C11 (PharMingen, San Diego, CA) and R-PE anti-mouse CD45R/ B220 clone RA3-6B2 (PharMingen). Briefly, splenocytes (1×10^6 cells) were washed and incubated with mAbs at 10 ug/ml in 0.1ml of staining medium: DMEM supplemented with 10%ECS, 10mM EDTA, 10mM

Hepes , and 0.1%NaN3 for 1 hour at 4C. After washing cells were fixed in 2% formaldehyde. All flow cytometric analyses were conducted using a FACScan (Becton Dickinson, Mountain View, CA).

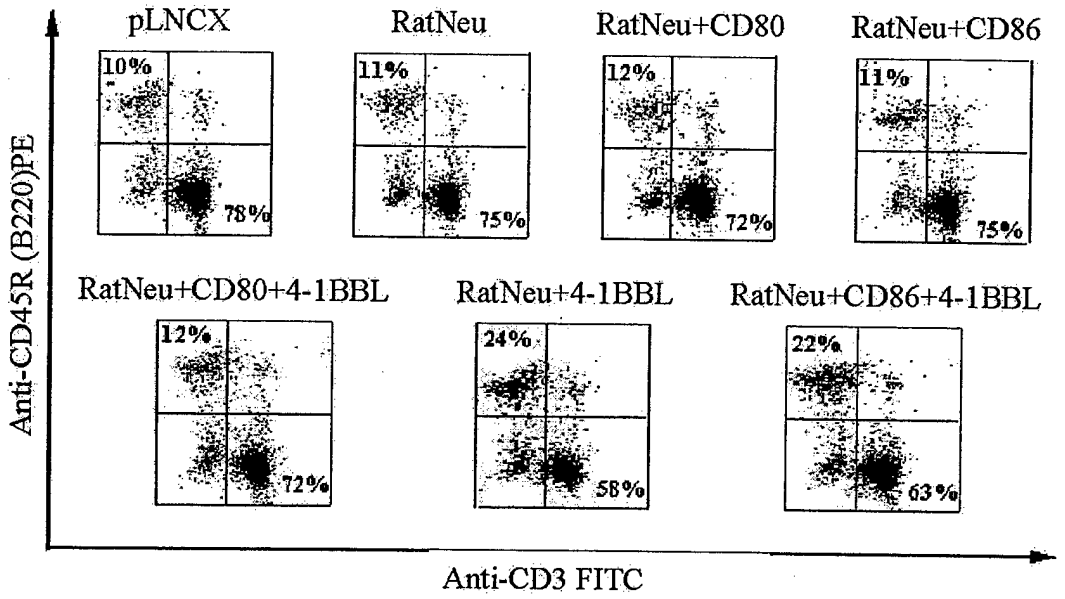


Figure 2 demonstrates the mean rat neu specific antibody levels for mice immunized with the various constructs (n=6/group). Animals immunized with pLNC+neu+CD86+4-1BBL demonstrated the highest levels of rat neu specific antibody.

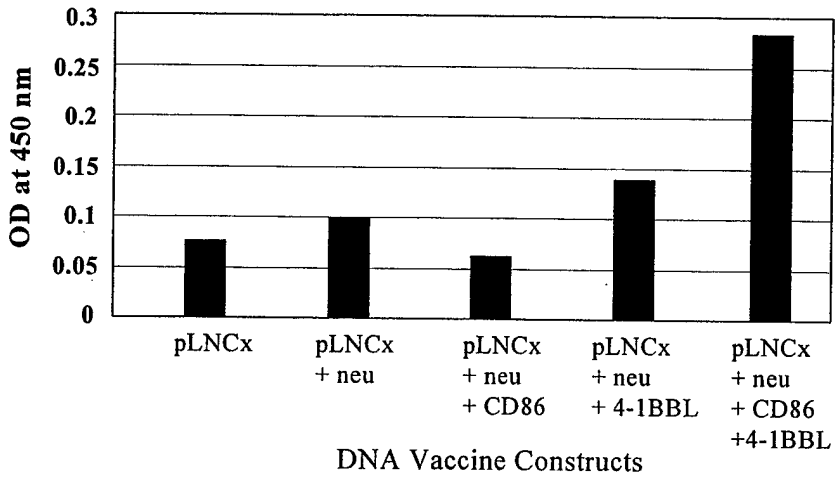


Figure 2. Mice immunized with HER DNA vaccines encoding CD86 and 4-1BBL develop significant levels of HER2 specific antibody. Sera from immunized mice were prepared and stored at -20°C until they were analyzed by ELISA for the presence of anti-rat neu (HER2) antibody. 96-well plates were coated with 2.5 ul/ml of c-neu (Ab-1) antibody (Calbiochem, Cambridge, MA) in 50 ul/well of carbonate buffer overnight at 4°C. The plates were incubated with a blocking buffer (PBS + 1% BSA) for 4 hrs at room temperature and washed with 0.05% Tween-20/PBS. Rows were alternatively coated with PBS + 1% BSA or DHFRG8 cell lysates 25 mg/ml, 50 ul/well, and incubated overnight at 4°C. After washing, the plates were incubated with 2-fold serial dilutions of sera in dilution buffer (PBS, 1% BSA, 1% fetal calf serum, 0.1NaN3 and 25 ug/ml mouse Ig (Organon Teknika, Durham, NC) for 1 hr at room temperature. Plates were then incubated for 1 hr at room temperature with a horse radish-conjugated goat anti-mouse IgG (diluted 1:5000 in PBS and 1% BSA) for antibody detection. After a final washing, the reaction was developed with TMB substrate buffer. Data is expressed as the OD at 450 nm.

Analysis of the lymph nodes draining the vaccination site demonstrates evidence of both vector as well as neu DNA indicating uptake and transport to the lymph node by local APC. In addition, FACs analysis shows the antibodies generated with these immunizations bound to intact tumor cells expressing neu on cell surface (data

not shown). Thus, the work performed in this aim has demonstrated that antibodies specific for ECD can be found in patient sera and function to inhibit growth of tumor cells (previous report). However, the incidence of these antibodies and level at which they are produced are low (previous report). Two methods to increase the incident and level of antibodies, protein as well as DNA vaccination have proven to be effective in animal models. Both these strategies will be further pursued for translation to human clinical trials.

Task 3: Months 1-48 To determine whether responses to HER2 are beneficial or detrimental, stratifying for function. These studies are ongoing and actively accruing patients. The studies require sera from large cohorts of breast cancer patients. The HER2 status needs to be known for all and all need to be treated with the same regimen. A major problem in breast cancer research in general and our work specifically, is a lack of sera from breast cancer patients linked to evaluable clinical databases. A study of antibody responses to HER2 will be performed in the context of NSABP B-27. Briefly, protocol B-27 is designed to determine whether 4 cycles of pre-operative or post-operative Taxotere given after 4 cycles of pre-operative Adriamycin (A) and cyclophosphamide (C) will more effectively prolong disease-free survival and overall survival than 4 cycles of pre-operative AC alone. Protocol B-27.1 has been designed to obtain and analyze serum from B-27 patients for the presence of HER2 circulating ECD and antibody and to correlate these factors with tumor response to pre-operative chemotherapy and survival.

Protocol B-27.1 requires patient blood to be drawn by the NSABP pre-therapy, post neo-adjuvant chemotherapy, post surgery, at 12 month follow up and at the first relapse. The sera is being processed and stored for evaluation of HER2 antibody. Both fresh frozen and paraffin-embedded tissue specimens are being collected and stored at the NSABP headquarters. The NSABP database includes information on demographics, risk factors, family history, clinical and pathologic factors, characteristics of tumor, treatment and outcome. Sera collection continues at the time of this progress report. Serial serum is beginning to be collected on the same individuals. All patients are receiving the same adjuvant chemotherapy regimen. Thus, the variable of HER2 reactivity can be analyzed. Stored sera will be examined in batches for Ab to whole HER2 protein, ICD and ECD. Circulating serum ECD levels will be measured. The level of HER2 overexpression on primary tumor will be determined by immunocytochemistry on tissue blocks by NSABP reference pathologists. The NSABP statistical group will provide correlations. As described above, assays have been standardized to be able to evaluate the study once the samples are released.

Task 4: Months 1-48 To determine whether Ab varies predictably with therapy and recurrence. Levels of antibody might correlate with important clinical parameters. Thus, following levels of Ab might provide information useful for decision making. When tumor progresses or relapses, the increase in HER2 antigen load might be expected to stimulate rising titers. A rise in Ab titer might serve as a harbinger of relapse. While the study described in Task 3, NSABP 27.1, will give prognostic information concerning the correlation of HER2 specific antibody response and survival, sufficient time points of sera will be collected that we may be able to discern whether HER2 antibody levels vary predictably with recurrence.

We have initiated collaboration with Dr. Saul Rivkin at the Tumor Institute at Swedish Hospital here in Seattle. Dr. Rivkin's group is responsible for the primary treatment and management of over 50 newly diagnosed breast cancer patients/year. In this study, we proposed to measure the antibody levels against HER2 in patients with HER2-positive breast cancer, stages I-IV, at different times during their disease course. All new patients with breast cancer, seen at the Tumor Institute, are approached to participate. Antibody levels are drawn at diagnosis and followed at 3 month intervals throughout the patient's treatment and follow-up. Correlation between HER2-specific antibody levels, treatment responses, disease progression, and prognosis will be assessed. The estimate time of follow-up is 3 years.

Task 5: Months 13-48 To determine whether immunity to HER2: (a) is present in patients with DCIS; (b) correlates with progression of HER2+ DCIS to HER2 negative invasive cancer; and (c) represents a possible marker for early cancer. Studies of breast cancer biopsies show that HER2 levels are increased in the majority of DCIS specimens, but are not seen in atypia or dysplasia. Thus, overexpression of HER2 appears to be associated with malignant transformation and early neoplasia, but not benign proliferative diseases of the breast. This observation raises the question whether HER2-negative invasive ductal breast cancer arises from HER2 positive DCIS and whether HER2 immunity plays any role in immunoselection of progressive HER2 negative invasive ductal cancer from HER2-positive DCIS. Evaluating the immune response of newly diagnosed patients with

DCIS and comparing that response to newly diagnosed patients with invasive breast cancer would lead to a better understanding of the interaction between the immune system and HER2 positive cancer.

The collaborations formed to collect material specifically on patients with DCIS include Dr. Carol J. Fabian at the University of Kansas Medical Center. Dr. Fabian is conducting a Phase I study of a new selective estrogen receptor modulator (SERM) through the Chemoprevention Branch of the NCI. Patients with DCIS, T1, and T2 breast cancer will receive the agent for the interval between initial diagnostic biopsy and subsequent definitive surgical resection. HER2 antibody levels will be assessed on these patients as well as HER2 overexpression in DCIS or invasive breast cancer. In addition, the group will collect sera on all patients with DCIS evaluated, even those who do not enter the study. It is hoped collecting samples within the context of a Phase I treatment study will increase the population available for sera collection. Secondly, we have started a study with the mammography tumor registry here at the FHCRC with Dr. Nicole Urban. Patients with abnormal mammogram and undergoing biopsy will have blood drawn at the time of biopsy. We will evaluate the level of HER2 antibody, as well as p53 antibody and c-myc antibody, in the blood and correlate the presence of an immune response to these oncogenic proteins with abnormal pathology.

Finally, sera from NSABP P-01 protocol provides a unique resource to address these issues. Protocol P-01 is designed to test the hypothesis that long-term treatment with tamoxifen is effective in preventing invasive breast cancer. Serum is being collected on 16,000 individuals. It is projected that 325 of the subjects will develop breast cancer over the next 8 years. By examining serial sera for Ab at the time of diagnosis of malignancy, at fixed future time points and at the time of entry onto the protocol it should be possible to determine whether immunity predates diagnosis. The prevalence of Ab to HER2 will be too low to provide a general screening assay for breast cancer.

Task 6: Months 1-48 To determine whether immunity to HER2 correlates with outcome of 2B1 bispecific Ab therapy. Task completed in 1998 report.

Specific Aim # 2: To examine CD4+ T cell immunity to HER2.

Task 7: Months 1-36 To develop *in vitro* priming with dendritic APC to generate HER2-specific CD4+ T cells and to identify the epitopes recognized. Task completed in 1999 report.

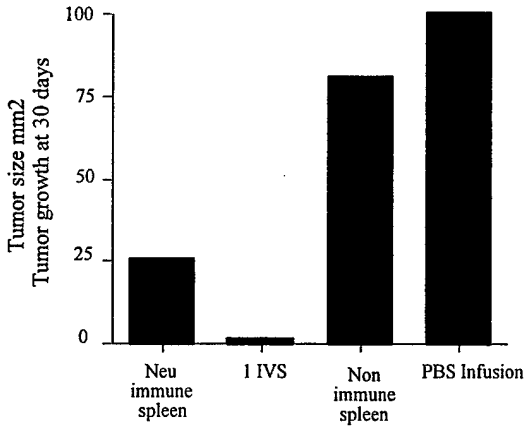
Task 8: Months 13-48 To determine whether Th immunity to HER2 can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2. Data presented in last year's progress report demonstrated that using APC such as dendritic cells, HER2 specific T cells could be expanded from the peripheral blood of patients without breast cancer. However, we have found that we can readily expand HER2 specific Th cells from patients after the immune responses have been augmented via immunization. The goal of this aim was to determine whether T cells could be expanded from patients in preparation for infusion for potential use in adoptive immunotherapy strategies. While T cells could be generated from patients who were not HER2 +, we found it much easier to isolate and expand T cells from patients who had been immunized and their precursor frequencies boosted. We began to expand HER2 specific CD4+ T helper cells from patients who had been immunized with a peptide based HER2 vaccine. After immunization, stimulation indices to HER2 protein ranged from 2.1-10.5. Expansion of T cells, as described below, through 2 IVS result in cultures 90% CD3+ with specific to HER2.

Task 9: Months 13-48 To determine the prevalence of CD4+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. Data collection is ongoing as described in Aim 1.

Task 10: Months 13-48 To determine whether CD4+ responses modulate the biology of autologous tumors *in vivo*. As described in last year's report, we have turned to an animal model of the neu-tg mouse to discern the function of various T cell subsets. Last years report demonstrated that animals immunized with a peptide-based vaccine could be protected form the development of HER2 overexpressing tumors. We developed methods to expand specific T cell subset from the mice after immunization to more completely explore the role of CD4+ and CD8+ T cells in eradicating disease. Animals were immunized with T helper epitopes derived from rat neu and challenged with a neu expressing syngeneic tumor. Spleens form those animals protected from the challenge were harvested and T cells further expanded in vitro with the immunizing helper epitope (15 mer) and IL-2. After

1 IVS the T cell lines were >85% CD3+ and 90% CD4+. T cells were infused into tumor bearing mice. Data demonstrates that CD4+ T cells specific for neu can result in an anti-tumor effect (Fig. 3).

Figure 3. Adoptive transfer of neu specific T cells can inhibit the growth of neu overexpressing tumor *in vivo*. Mice were immunized with a neu peptide vaccine, 2 immunizations 14 days apart. Spleens were harvested from immune animals and infused into tumor bearing animals or enriched through 1 *in vitro* stimulations with the immunizing neu peptides and then infused. Animals were implanted with $2x10^6$ neu syngeneic tumor cells on day 1, and on day 2 infused with $2x10^7$ cells derived from immune spleen, immune spleen after 1 IVS with neu antigen, spleen from non-immunized syngeneic mice, or PBS. Data is expressed as the mean of 3 mice/group of tumor measurements taken 30 days after implant.



Thus, CD4+ T cells, specific for neu helper peptides can modulate the growth of neu expressing tumors *in vivo*.

Specific Aim # 3: To examine CD8+ CTL immunity to HER2.

Task 11: Months 1-48 To develop *in vitro* priming with dendritic APC to generate HER2 specific CD8+ T cells and to identify the epitopes recognized. Task completed in 1999 report.

Task 12: Months 1-36 To determine whether CD8+ CTL immunity can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2. Task completed in 1999 report. Again, the use of DC to generate neu specific CTL has been defined. However, expansion methods to generate large numbers of specific T cells suitable for therapy is more feasible when patients have been immunized. We have demonstrated, in previous reports, that precursor frequencies can be boosted after immunization. In the final analysis of 20 patients immunized with HER2 peptide vaccines consisting of HLA-A2 binding motifs, the average peptide specific frequency obtained was 1:19,000 PBMC as defined by IFNg release via ELISPOT (range <1:100,000 to 1:3,000). T cells clones from patients after immunization demonstrated that the peptide specific T cells could lyse HER2 overexpressing tumors as well (Fig. 4).

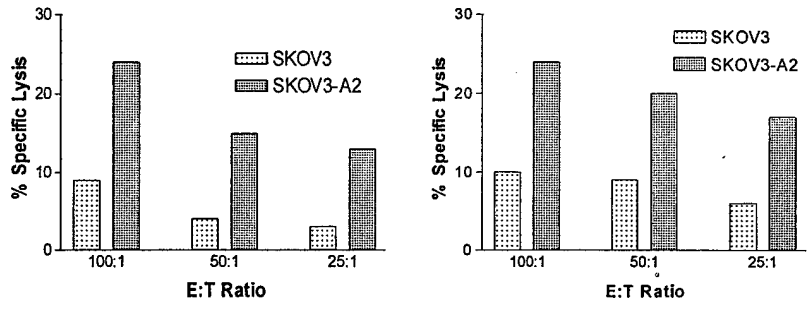


Figure 4. HER2 peptide specific T cells lyse tumors that are HLA matched and overexpress HER2. T cell clones were isolated from a patient undergoing vaccination. The clones were tested for tumor cell killing in a 4-hour chromium release assay. The targets used were the HER2 overexpressing cell lines

SKOV3 and SKOV3-A2. SKOV3-A2 are SKOV3 cells transfected with and expressing HLA-A2. Results are expressed as the average % specific lysis from six replicates. Results shown are from two independently derived clones and are representative of greater than 20 p369-9mer specific clones isolated.

Thus, HER2 specific CD8+ T cells can be isolated form patients with HER2 expressing breast cancer.

Task 13: Months 37-48 To determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. The role of HER2 specific T cell immunity can be addressed in terms of evaluating an animals models as described in Aim 2 for the CD4+ T cells or can be addressed in terms of a Phase I clinical trial of infusion of HER2 specific T cells. We developed cytokine strategies for the expansion of polyclonal T cell lines, specific for HER2, after immunization. Of foremost consideration when performing *ex vivo* expansion of T cells is establishing the appropriate culture conditions that allow for rapid expansion while preserving antigen specificity and CTL activity.

IL-7 will increase cell number with a loss of antigen specificity. IL-7 has previously been reported to be a useful cytokine for preserving antigen-specific CTL function in T cells cultures. However, as shown in Figure 5, while IL-7 enhances cell number expansion (Fig. 5A), its use results in the loss of specificity with a concomitant increase in background non-specific lysis (Fig. 5B). Example shown is representative of several patients studied in this manner. Studies in murine models have previously reported that T cells can be adversely effected by long term culture with IL-2 and IL-7, but not IL-2 and IL-4.

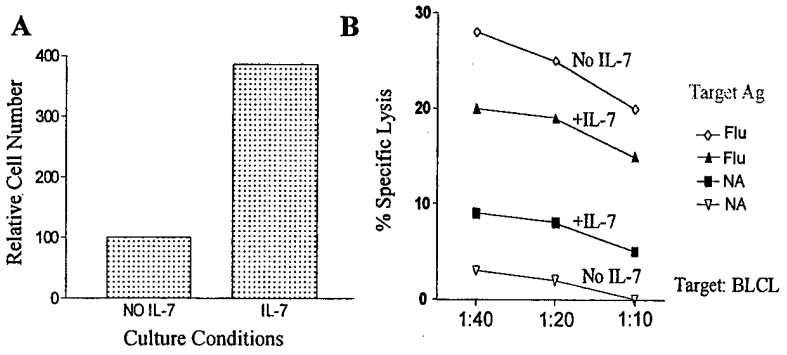


Figure 5. IL-7 will increase cell number with a loss of antigen specificity. PBMCs were isolated from donor peripheral blood. The PBMCs were expanded for 12 days in the presence of IL-7 (25/ng/ml). IL-7 was added day 0. IL-2 was added to 10 U/ml on day 5 and day 10. On day 14 the PBMCs were washed, enumerated (**A**), and subjected to a 4-hr chromium release assay (**B**) using BLCLs, homozygous HLA-A2 B cells, as targets. Results are expressed as the average % specific lysis from six replicates.

However, IL-12 will increase cell number with preservation of specificity. Figure 6 demonstrates that in contrast, when IL-12 was added to culture along with the IL-2 both cell number (Fig. 6A) and lytic activity was enhanced (Fig. 6B). Example shown is representative of several patients studied in this manner. In another antigen system, flu matrix, IL-12 was evaluated for the ability to selectively expand antigen-specific precursors to HLA-A2 binding peptide, GILGFVFTL. As shown in Figure 7, the addition of IL-12 to IL-2 in the presence of antigen resulted in a 3-fold increase in precursor frequency.

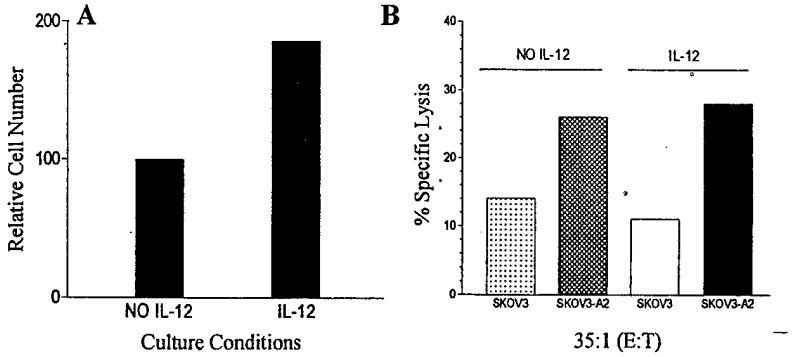


Figure 6. IL-12 will increase cell number with preservation of specificity. PBMCs were isolated from peripheral blood of a patient undergoing vaccination with HER2 peptides. The PBMCs were expanded for 12 days in the presence of IL-12 (10/ng/ml). IL-12 was added day 0 and again at day 5. IL-2 was added to 10 U/ml on day 5. On day 10 the PBMCs were washed, enumerated (**A**), and subjected to a 4-hr chromium release assay (**B**) using SKOV3 and SKOV3-A2 as targets. SKOV3-A2 are SKOV3 cells transfected with and expressing HLA-A2. Results are expressed as the average % specific lysis from six replicates.

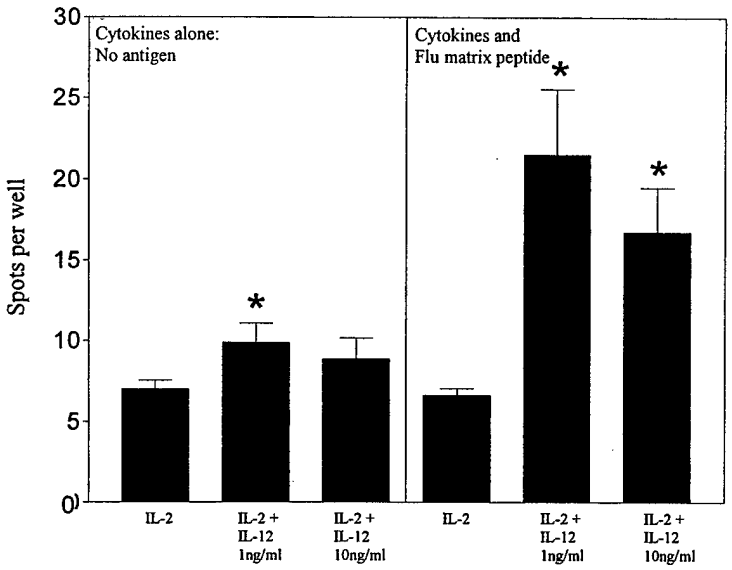


Figure 7. IL-12 enhances antigen-specific $CD8^+$ T lymphocyte precursor frequencies to flu matrix peptide after 1 IVS. PBMC from a normal HLA-A2 donor were plated in 6 groups at 20 x 10^6 cells per group in 20 mls RPMI-10% AB. Flu matrix peptide (10ug/ml) was added to half the cultures on day 0. On day 4, IL-2 was added to all groups to 10 U/ml. On day 4 also, IL-12 was added to particular cultures at either 1 ng/ml or 10 ng/ml concentration. On day 8, IL-12 and IL-2 were added as on day 4. On day 12, the cells were counted and plated in 96-well plates in 8 replicates/group at 2.5 x 10^5 cells/well. To each well, 2.0 x 10^5 irradiated autologous, irradiated (3000 rad) PBMC were added along with flu matrix peptide (10ug/ml). The cells were incubated for a further 20 hrs, resuspended in fresh medium, and transferred to an IFN coated 96 well plate for ELIspot analysis. ELIspot was carried out as previously described. Data presented are given as the mean (±SEM, *=$p \leq 0.03$) number of spots per well for the 8 replicates.

Thus, data presented in this aim demonstrates that human HER2 specific T cells can be readily expanded (previous report 1999) and retain functionality by minimal *in vitro* manipulations after active immunization. These studies have resulted in the initiation of a Phase I clinical study of the infusion of HER2 specific T cells into patients with HER2 overexpressing cancers for the treatment of refractory disease.

KEY RESEARCH ACCOMPLISHMENTS, 1999-2000

- Developed a HER2 and p53 antibody assay within CLIA standards suitable for the analysis of large-scale trials.
- Developed a rapid ELISA based method using his-tag recombinant proteins to evaluate the incidence of immunogenicity of novel breast cancer antigens.
- Generated DNA vectors suitable for immunizing to generate antibody immune responses specific for the ECD of HER2.
- Constructing HER2 proteins for use in human clinical trials (CORIXA)
- Continue collection of sera on established clinical trials for analysis when statistical considerations have been met or when the trials have matured.
- CD4+ HER2 specific T cells are readily expanded after precursor frequencies have been boosted by active immunization of cancer patients.
- Neu specific CD4+ T cells can mediate an anti-tumor response in the neu-tg mouse.
- CTL specific for tumors expressing HER2 can be generated form the PBL of cancer patients.
- HER2 specific CD8+ T cells can be readily expanded from the PBL of immunized patients and retain functionality laying the foundation for a clinical trial of infusion of HER2 specific T cells.
- Initiation of a Phase I trial of a HER2 protein based vaccine. Trial designed, IND written, passed by regulatory agencies, funding secured from the NCI.
- Initiation of a Phase I trial of the infusion of HER2 specific T cells in patients with advanced stage cancers. Trial designed, IND written, passed by regulatory agencies, funding secured from the NCI.

REPORTABLE OUTCOMES:

BIBLIOGRAPHY

(These references all acknowledge DAMD as support of work published. All references listed from the start of the funding period, 1997-2000)

1. Disis, M.L., Pupa, S.M., Gralow, J.R., Dittadi, R., Menard, S., and Cheever, M.A.: High titer HER-2/neu protein specific antibody immunity can be detected in patients with breast cancer. J. Clin. Oncology. 15 (11): 3363-3367, 1997.
2. McNeel, D.G., Schiffman, K.A., and Disis, M.L.: Immunization with rhGM-CSF as a vaccine adjuvant elicits both a cellular and humoral response to rhGM-CSF. Blood. 93: 2653-2659, 1999.
3. Ward, R.L., Hawkins, N.J., Coomber, D., and Disis, M.L.: Antibody immunity to the HER-2/neu protein in patients with colon cancer. Human Immunology. 60:510-515, 1999.
4. Disis, M.L., Grabstein, K. H., Sleath, P. R., and Cheever, M.A.: Generation of immunity to the HER-2/neu oncogenic protein in breast and ovarian cancer patients using a peptide based vaccine. Clin Cancer Research. 5:1289-1297, 1999.
5. Disis, M.L., Schiffman, K., Gooley, T.A., McNeel, D.G., Rinn, K., and Knutson, K.L.: Delayed type hypersensitivity response (DTH) is a predictor of peripheral blood T cell immunity after HER-2/neu peptide immunization. Clinical Cancer Research. 6(4):1347-50, 2000.
6. McNeel, D.G., Nguyen, L.D., Storer, B., Vessella, R., Lange, P.H. and Disis, M.L.: Antibody immunity to prostate cancer-associated antigens can be detected in the serum of patients with prostate cancer. Journal of Urology (in press), 2000.
7. Disis, M.L., Knutson, K.L., Schiffman, K., Rinn, K., and McNeel, D.G.: Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Research and Treatment. 1788:1-8, 2000.
8. Disis, M.L. and Cheever, M.A.: HER-2/neu protein: A target for antigen specific immunotherapy of human cancer. Adv in Cancer Research, Vande Woude, G.F. and Klein, G. (Eds), Academic Press, San Diego, U.S.A., 71: 343-371, 1997.
9. Disis, M.L. and Cheever, M.A.: HER-2/neu oncogenic protein: Issues in vaccine development. Critical Reviews in Immunology. 18:37-45, 1998.
10. Schoof, D.D., Smith, J.W., Disis, M.L., Brandt-Zawadski P., Wood, W., Doran, T., Johnson, E., and Urba, W.J.: Immunization of metastatic breast cancer patients with CD80-modified breast cancer cells and GM-CSF. Advances in Experimental Med. Biol. 451: 511-518, 1998.
11. Disis, M.L., McNeel, D.G., Schiffman, K.A., Rinn, K. and Knutson, K.L.: Peptide based tumor vaccines. Current Opinion in Oncologic, Endocrine, and Metabolic Drugs. 1(3): 253-259, 1999.
12. Knutson, K.L., Schiffman K., Rinn, K., and Disis, M.L.: Immunotherapeutic approaches to the treatment of breast cancer. J. Mammary Gland Biol. and Neoplasia, (4):4; 353-365, 1999.
13. Disis, M.L. and Schiffman, K.: Cancer vaccines targeting the HER-2/neu oncogenic protein. (in press) Seminars in Oncology, 2000.
14. Knutson, K.L. and Disis, M.L.: Expansion of HER-2/neu specific T cells ex vivo following immunization with a HER-2/neu peptide based vaccine. (in press), Biological Therapy of Breast Cancer, 2000.

Manuscripts Submitted:

1. Knutson, K.L. and Disis, M.L.: IL-12 Enhances in vitro expansion and function of peptide-specific T lymphocytes. Submitted, 2000.
2. Schiffman, K., Rinn, K., and Disis, M.L.: Delayed type hypersensitivity (DTH) response to recall antigens does not accurately reflect immune competence in advanced stage cancer patients. Submitted, 2000.
3. Disis, M.L., Shiota F.M., and McNeel, D.G.: Rat neu specific cytotoxic T cells can be generated by DNA immunization with soluble cytokine as an adjuvant. Submitted, 2000.

Abstracts (last 2 years only):

1. Knutson, K.L. and Disis, M.L.: Isolation of HER-2/neu specific gamma-delta TCR T cell clones from an HLA-A2 ovarian cancer patient immunized with a HER2 peptide-based vaccine. FASEB, 2000.
2. Disis, M.L., Gooley, T.A., Knutson, K.L., Cheever, M.A. Rinn, K., Davis, D. and Schiffman, K.: Phase I

study of HER-2/neu peptide based vaccines for the generation of HER2 specific immunity in patients with HER2 overexpressing cancers. Proceedings of ASCO, 2000.

3. Schiffman, K., Rinn, K., Gooley, T.A., and Disis, M.L.: Delayed type hypersensitivity (DTH) response is a predictor of peripheral blood T cell immunity after HER2 peptide immunization. Proceedings of ASCO, 2000.
4. Goodell, V., Schiffman K., and Disis, M.L.: Analysis of HER-2/neu specific antibody immunity generated in patients with HER2 expressing cancers following immunization with peptide based vaccines. Proceedings of ASCO, 2000.
5. Smorlesi, A., Shiota, F.M., and Disis, M.L.: A murine neu-transgenic (neu-tg) breast cancer tumor cell line established *in vitro*, expresses Class I and II MHC and retains expression *in vivo*. Proc. Amer. Assn Canc. Res. 41:512, 2000.
6. McNeel, D.G., Nguyen, L.D., and Disis, M.L.: T-cells, derived from patients with prostate cancer, secrete IFNγ in response to stimulation with prostate cancer antigens. Proc. Amer. Assn Canc. Res. 41: 5593, 2000.
7. McNeel, D.G., Nguyen, L.D., and Disis, M.L.: Identification of potential CD4+ T helper epitopes derived from the protein sequence of prostatic acid phosphatase (PAP). Proc. Amer. Assn Canc. Res. 41:4444, 2000.
8. Knutson, K.L. and Disis, M.L.: IL-12 enhances *In Vitro* expansion and function of peptide-specific T lymphocytes. Proc. Amer. Assn Canc. Res. 41:1236, 2000.
9. Goodell, V. and Disis, M.L.: Application of CLIA standards to assays for immunologic monitoring of cancer vaccine trials. Proc. Amer. Assn Canc. Res. 41:4025, 2000.
10. Crosby, P.J. and Disis, M.L.: Cryopreservation dampens antigen-specific T-cell responses: Implications for monitoring cancer vaccine trials and corrective protocols. Proc. Amer. Assn Canc. Res., 41:4026, 2000.
11. Disis, M.L., Grabstein, K.H., Sleath, P.R., and Chevver, M.A.: HER-2/neu peptide vaccines elicit T cell immunity to the HER-2/neu protein in patients with breast and ovarian cancer. Proceedings of ASCO, 17:97, 1999.
12. McNeel D.G. and Disis M.L.: T cell immunity specific for prostatic acid phosphatase (PAP) results in a destructive prostatitis in a rodent model. Proc. Amer. Assn Canc. Res., 40: 256, 1999.
13. McNeel D.G., Vessella R., Lange P.H. and Disis M.L.: Antibody immunity to prostate cancer antigens can be detected in patients with prostate cancer. Proc. Amer. Assn Canc. Res., 40: 353, 1999.
14. Knutson K.L., Schiffman K., and Disis M.L.: Generation of antigen-specific CD8+ T lymphocytes following immunization with helper epitopes of HER-2/neu. Proc. Amer. Assn Canc. Res., 40:576, 1999.
15. Knutson K.L., Crosby P.J., and Disis M.L.: Isolation of HER-2/neu (HER2) specific T cell clones from a breast cancer patient following immunization with a HER2 peptide vaccine. Proc. Amer. Assn Canc. Res., 40:310, 1999.
16. Smorlesi A., Shiota F., Disis M.L.: Flt3-Ligand as a vaccine adjuvant, results in the generation of antigen specific IFNγ producing T cells after immunization. Proc. Amer. Assn Canc. Res., 40: 313, 1999.

FUNDING APPLED FOR BASED ON WORK SUPPORTED BY THIS AWARD

Funded:

R01, *HER2 Peptide Vaccines in Patients with HER2 Expressing Cancers*, NCI, 1997-2001, Funding: $107,547/yr

SPORE in Ovarian Cancer, NCI, 1999-2004, Funding: $107,410/yr (co-PI and Project V)

R01, *Phase I Trial of a Peptide Vaccine against EGFRvIII*, 1999-2002, NCI, Funding: $156,151/yr

R01, *Novel Vaccine Targets for Early Stage Breast Cancer*, 2000-2003, NCI, Funding: $208,974/yr

R01, *HER2 Specific T Cell Infusions Following HER2 Peptide Based Vaccination for the Treatment of HER2 Overexpressing Malignancy*, 2000-2005, NCI, Funding: $335,000/yr

K24, *Midcareer Investigator Award in Patient Oriented Research*, 2000-2005, NIH, Funding: $107,888/yr

Submitted:

P01, Mechanisms and Markers of Prostate Cancer Metastases, 2000-2005, NCI, Funding: $193,982/yr (Project V)

U54, Immunologic Correlates of Effective Immunization for Cancer Vaccines, 2001-2006, NCI, Funding: $1,300,000/yr (P.I., Admin Core, Specimen Core, Project IV)

EMPLOYMENT

Salaries of the personnel that worked on the projects listed for no less than 1 year.

University of Washington:	Mary L. Disis, M.D. (no salary used, was funded on a K08) PhD/Professional Staff: Kevin Whitham, Keith Knutson Technicians: Paul Crosby, Lan Nguyen, Vivian Goodell
Corixa:	Martin Cheever, M.D. PhD Staff: Gary Fanger, Teri Foy, Robert Henderson, Nancy Hosken, Aijun Wang Technicians: Lisa Anest, Jeanette Bannink, Patrick McGowan, Kimberly Stankey

CONCLUSIONS:

The overall goal for the proposal was to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. These goals have been accomplished by the end of the funding period. The studies have validated that immunity to HER2 exists and can be augmented by manipulation *in vitro*. The studies have thus far resulted in a Phase I study of a HER2 peptide-based vaccine and a Phase I trial of HER2-specific adoptive immunotherapy.

Studies of antibody responses confirmed that Ab immunity to HER2 can be detected in the sera of some patients with breast cancer and are correlated with antigen expression. The increased frequency of Ab in patients with HER2+ cancer strongly implies that immunity develops as the result of the overexpression of HER2 on breast cancer, i.e., some patients become immune to their own cancers. We now know the prevalence of HER2 antibody in patients with early and advanced stage HER2 breast cancers. In addition, we are developing strategies specifically to increase these antibody responses. These vaccination strategies will be translated to human clinical trials of breast cancer vaccines. HER2 is a functioning growth factor receptor. Ab to HER2 in some patients was directed against the ECD and was able to perturbate function. This remarkable finding strongly implies that the immune response to HER2 might directly alter growth characteristics and outcome in patients with breast cancer. These studies may eventually provide evidence of substantial and important host tumor interactions. The correlation between antibody and breast cancer shows that antibody responses have the potential to serve as tumor markers for detecting breast cancer. That hypothesis is being pursued. Also being pursued is the hypothesis that changes in level of Ab can detect early relapse. The major question of whether existent immunity to HER2 relates to improved survival is being addressed using sera collected from the NSABP adjuvant breast cancer trials. Sera are drawn at the time of diagnosis and all patients are receiving the same adjuvant chemotherapy regimen. Thus, HER2 reactivity can be analyzed as an independent variable. Finally, HER2 immune sera provides an excellent reagent to screen for other novel breast-related immunogenic proteins and 5 potential new breast cancer antigens have already been identified. Large-scale clinical analysis of antibody responses to immunogenic oncogenic proteins can take place due to the development of clinical grade assays.

Studies of CD4+ T cell immunity to HER2 confirmed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy given that HER2 is an abundant soluble protein, i.e., the extracellular domain (ECD) is shed. In animal models CD4+ T cells can be effective against abundant soluble proteins. In fact, in our biologically relevant animal model, the neu-tg mouse, infusion of HER2 specific T helper cells resulted in an anti-tumor response. We have developed highly sensitive and reproducible assays for evaluating CD4+ and CD8+ T cell responses to HER2. These assays are being used in the current clinical trials to identify, quantify and follow existent immune responses to HER2. We are able to generate HER2 specific CD4+ T cells *in vitro* and have developed a clinical trial to determine the role CD4+ T cell immunity plays in clinical response.

Studies of CD8+ T cell immunity to HER2 confirmed that CD8+ CTL could be primed to HER2 peptides *in vitro* and that primed peptide specific CTL can lyse HER2 positive cancer cells. We have shown studies that determine the most clinically efficient, physiologically relevant, means of generating HER2-specific CTL by priming *in vitro*. A major issue is whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor. We have been able to generate HER2 specific lines and clones and characterize them as to their potential function. Finally, we have determined that HER2 specific T cells can be expanded with maintenance of function to the extent presumed necessary for therapy.