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Martyn G. Chamber 11/18/87
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INTRODUCTION:

The HER-2/neu (H2N) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. H2N over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. H2N 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 is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against H2N. 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 H2N. H2N is a self protein. Therefore, before our studies it had been assumed that patients would be immunologically tolerant to H2N 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 H2N is induced in some individuals by virtue of the presence of growing cancer expressing the antigen and gives credence to the concept that H2N-specific immunity can potentially be used in therapy without destroying normal tissue. The current grant is exploring issues important for developing H2N specific vaccines and T cell therapy. In addition, the demonstration of immunity to H2N is offering the opportunity to explore host-tumor interactions in a well-defined antigen system.

Specific Aim #1 is examining Ab immunity to H2N. Preliminary data showed that Ab immunity to H2N can 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 H2N are beneficial or detrimental. Additional studies were proposed to determine whether immunity to H2N 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 H2N. Preliminary data showed that some patients with H2N-positive breast cancers exhibit primed CD4+ helper T cell responses to H2N. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy. In animal models CD4+ T cells can be effective against abundant soluble proteins. The extracellular domain (ECD) of H2N is shed abundantly in some patients. Studies were proposed to determine the prevalence of CD4+ responses in patients with H2N+ tumors and to determine whether changes in immunity occur with therapy and relapse.

One limitation to the development of human anti-cancer vaccines and T cell therapy is that determination of immunogenicity conventionally requires immunization in vivo. Therefore, much effort was proposed to develop methods of priming in vitro. Preliminary data in the grant proposal described prior studies developing a culture system using dendritic antigen presenting cells that allows exceedingly rapid priming in vitro. Studies were proposed to determine whether the priming system is reproducible enough and powerful enough to allow determination of which peptides are immunogenic and which peptide specific T cells are capable of responding to whole H2N protein. Identification of epitopes is important for eventual incorporation into peptide based vaccines or for use to stimulate T cells in vitro for T cell therapy regimens.

Specific Aim # 3 is examining CD8+ CTL immunity to H2N. Preliminary data for the grant showed that CD8+ CTL can be primed to H2N peptides in vitro and that primed peptide specific CTL can lyse H2N 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 H2N. Additional studies were proposed to determine whether patients with breast cancer have existent CTL immunity to H2N, as had previously been described for patients with ovarian cancer. For patients with CTL immunity and H2N positive cancers, studies were proposed to determine the prevalence of CD8+ responses in patients with H2N+ tumors and the evolution of immunity with therapy and relapse. Finally, studies were proposed to determine whether H2N-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.
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 HER-2/neu.**

Task 1: Months 1-48 To determine the frequency of Ab, the biologic function of H2N specific Ab, Ab correlation with H2N overexpression and Ab correlation with circulating ECD.

Determining the frequency of Ab to H2N required first obtaining adequate sera from a well defined patient populations. Prior studies had been performed with sera drawn at random times in the breast cancer course. Several studies are currently underway analyzing sera drawn at the time of diagnosis in well characterized patients. Two studies have been completed to date. The first addressed the incidence of H2N antibody response in newly diagnosed breast cancer patients who were at different stages of disease. We established a collaboration with Drs. Serenella M. Pupa and Sylvie Menard from the Division of Experimental Oncology E, Instituto Nazionale Tumori, Milano, Italy. The Milano group provided sera from 127 breast cancer patients. The patients were characterized as to H2N protein overexpression in primary tumor and stage of disease. The sera was analyzed for the presence of H2N specific antibodies with a capture ELISA utilizing a H2N specific monoclonal antibody. A human breast cancer cell line (SKBR3) overexpressing H2N was used as the source of H2N protein. Sera from 200 volunteer blood donors was used as a control population.

The presence of H2N specific antibody responses correlated with the presence of breast cancer. Antibodies at titers of > 1:25 were detected in 25 of 127 (20%) breast cancer patients versus 2 of 200 (1%) normal controls. The specificity of antibody responses for patients with breast cancer increased when analyzed for higher titer responses. Antibodies at titers of >1:100 were detected in 14 of 127 (11%) breast cancer patients versus 0 of 200 (0%) normal controls. For early stage disease, the presence of higher titer antibody correlated with the presence of H2N expression by the primary cancer. Antibodies at titers of >1:100 were detected in 9 of 39 (23%) of patients with H2N positive stage I/II breast cancer versus 3 of 56 (5%) patients with H2N negative stage I/II breast cancer (p=0.03). In patients with advanced stage breast cancer the incidence of antibody to H2N was less than for early stage disease and there was no association between antibody to H2N and the expression of H2N by primary cancer. The lack of H2N specific antibodies in advanced stage patients can not be explained solely by immunosuppression associated with malignancy as the advanced stage population had antibody responses to recall antigens of the same magnitude as normal blood donors (Fig. 1). Antibody responses could be substantial. Titers of >1:12,800 were detected in 5 of 95 patients with early stage disease.

A second completed study was performed on sera from pre-menopausal women diagnosed with breast cancer in Western Washington (WISH study) in collaboration with Drs. Janet Dahling and Kathi Malone, from the Fred Hutchinson Cancer Research Center (FHRC). This study was undertaken to ascertain breast cancer risk factors that may correlate with antibody response. The FHRC has maintained a population based breast cancer registry. Significant epidemiologic data has been collected on all women less than 45 years old in Western Washington who have been diagnosed with invasive breast cancer from 1983 to present. Serum for analysis was available on the majority of these patients. Sera was identified from 188 patients who were newly diagnosed and had no other intervention than surgery. These sera were evaluated for the presence of H2N antibodies. Antibodies to the H2N protein were found in 16% (30 patients) of the study population. Tumors have not yet been analyzed for H2N protein overexpression. It is known that the extracellular portion (ECD) of the HER-2/neu protein can become truncated from the protein and circulate in the patient's sera. Measurements of circulating ECD have been shown by other investigators to be a measure of tumor bulk. The presence of circulating antigen may significantly effect the ability of an antibody to functionally interact with a tumor. Antibodies may bind circulating antigen and, thereby, enhance antibody clearance. The interaction of circulating ECD and serum antibody was explored by evaluating circulating ECD in 176/188 patients. 6/176 patients had detectable levels of circulating ECD protein. 3/6 had a detectable H2N antibody response and 3/6 had no evidence of antibodies to H2N. Thus, no conclusions can be made about the interactive role of anti-HER-2/neu antibody and circulating ECD. Interview data pertaining to demographics, family history and risk factors.
were available on these women. In addition, many of these patients have had hospital record reviewed for data concerning pathology, histology, stage of tumor, ER/PR status, DNA ploidy studies and treatment. The presence of HER-2/neu specific antibodies was compared to known risk factors. The presence of antibodies correlated to limited disease, e.g. T1 or T2, NO tumors (p<0.01). Specifically, the presence of H2N antibodies correlated with a lack of lymph node metastasis. There was no correlation with age at diagnosis or family history of breast cancer. There was no correlation with disease outcome which may be due to the limited number of patients from this population who have died during the course of the study.

Quantitation of the H2N specific antibody response may allow clinical correlations to be made in terms of the magnitude of H2N specific immunity. Although antigen specific antibody responses are typically reported as a serum titer, antibody responses can be more precisely quantified by incorporation of a standard curve into the assay. Figure 2 shows data from 3 H2N positive breast cancer patients who have antibody responses directed to H2N. The patient designated as AS had an antibody titer of 1:200. The patients designated as VB and LB both had antibody titer 1:100. The quantitative ELISA is a sandwich assay similar to the H2N antibody assay described above, except that wells set up as a standard curve are incubated with purified human IgG, IgM or IgA (Sigma BioSciences, St. Louis, MO) in serial dilutions ranging 0.5 μg/well to 0.008 μg/well. Rows of wells are coated with alternating PBS/1% BSA and lysates of SKBR3, a breast cancer cell line which overexpresses the H2N protein. Human sera is analyzed at 4 dilutions. The μg/ml values of H2N specific IgG for each patients is shown as well as the mean and 3 standard deviations of the value of 50 normal controls. Values are calculated in μg/ml based on a standard curve generated by four parameter analysis.

Recent experiments demonstrate that H2N immune sera can also be used to define novel tumor antigens. Figure 3. shows immune sera from patients with breast cancer were used to detect novel immunogenic proteins from lysates derived from SKBR3, a H2N overexpressing breast cancer cell line. Briefly, lysates were prepared from 10^7 SKBR3 cells and proteins separated by SDS PAGE (7% gel). After transfer to nitrocellulose (Hybond-C, Amersham) the blot containing tumor cell derived proteins was cut into strips and proteins were identified by immunoblotting with human sera as primary antibody. Human sera was used in a 1:500 dilution with TBS/1% BSA and 0.1% NP-40. A polyclonal antihuman-HRP conjugated second antibody (Amersham) was used in a 1:10,000 dilution. The blot was then developed using a chemiluminescent reaction (Amersham ECL). Western blots using sera from 9 breast cancer patients and 1 normal blood donor are shown in Figure 3. Patients 1, 2, 5, 8, and 9 are known to have H2N specific antibody immunity and serve as a positive control. These patients have detectable bands at p185 (molecular weight of the H2N protein). As an example, a unique band at approximately 100 kD could be detected in 7/9 breast cancer patients, but not in the normal control sera. Arrows demonstrate some unique bands that are seen after blotting with cancer patient sera but not with normal sera. N-terminus sequencing of these novel immunogenic proteins is pending.

The presence of H2N specific antibody responses in breast cancer patients and the correlation with H2N positive cancer in early stage patients strongly imply that immunity to H2N develops as a result of exposure of patients to H2N protein expressed by autologous cancer. The correlation between antibody and breast cancer shows that antibody responses have the potential to serve as a tumor marker for detecting the breast cancer. More accurate means of measuring antibody, such as direct quantitation, may allow improved sensitivity and specificity of serum H2N antibodies as a potential diagnostic or prognostic tool. The most optimistic interpretation of the finding that immunity correlates with early stage disease is that the development of an immune response to H2N expressed by growing tumor limits further growth and metastasis. Finally, sera from breast cancer patients can be used to define potentially novel breast cancer antigens. These findings should stimulate further studies to develop the use of immunity to oncogenic proteins as tumor markers and should stimulate the development and testing of vaccine strategies to induce and augment immunity to H2N for the treatment of breast cancer.

Task 2: Months 1-24 To examine biologic function of Ab binding to the ECD.

H2N is a growth factor receptor. Molecules binding to the H2N receptor, including the putative natural ligand(s), can have variable effects on stimulating malignant cells.
Heterologous antibodies can have an array of effects upon binding to the H2N extracellular region, with some inducing agonistic effects and some antagonistic effects. In order to evaluate and subsequently predict whether patient H2N Ab correlates with clinical outcome, it will be necessary to determine whether some Ab is function. If some Ab is functional, in order to study the correlation between Ab and outcome, it will be necessary to stratify patients as to whether their Ab is functional and the nature of that function. Preliminary data showed that several patients had Ab to the cysteine rich area of the ECD and, in one of the patients, that the Ab induced increased protein phosphorylation of H2N+ breast cancer cells. To fully characterize functional H2N antibodies recombinant polypeptides corresponding to the ICD and ECD domain proteins of H2N have been constructed. H2N ICD and ECD were separately cloned by PCR from c-erbB-2 cDNA, (provided by Jacelyn Pierce, Ph.D., NCI). Both domains are expressed in E. coli using a pET vector with an amino-terminal poly His tag. The expression of polypeptide is induced with IPTG and E. coli pellets harvested after an additional 4 hours. Recombinant ICD was purified from inclusion bodies by a combination of Ni^{2+} affinity chromatography, size exclusion, and ion exchange chromatography. The purified ICD is greater than 95% pure as judged by Coomassie Blue staining of protein gels and Western blot analysis. The recombinant polypeptide is also free of endotoxin. Recombinant ECD was purified from inclusion bodies by Ni^{2+} affinity chromatography alone and exhibits a similar degree of purity as the ICD protein. Eukaryotic vectors producing the ICD and ECD polypeptides are also being constructed. Post translational modification and protein folding necessary for human antibody recognition of H2N ICD and ECD may require eukaryotic rather than prokaryotic protein production. ICD and ECD specific antibody levels are determined using indirect ELISA with recombinant polypeptides. The use of recombinant domain proteins has allowed a more rapid analysis of patients with ECD specific responses. The function of the Ab in those patients has been further assessed in a more quantitative fashion. Data from a patient whose purified Ig inhibited H2N phosphorylation is shown as an example.

One measure of H2N function is tyrosine phosphorylation of the H2N ICD. Figure 4 demonstrates cancer cells overexpressing the H2N protein have decreased levels of H2N specific phosphorylation after incubation with human sera containing ECD specific antibodies. Sera was obtained from a patient with an ECD specific antibody response. Ig was purified from the sera by separation over an agarose A column. C-neu-5 is a H2N ECD specific monoclonal antibody that has been shown to increase phosphorylation of neu tyrosine kinase when it is incubated with H2N positive tumor cells and was used as a positive control. Phosphorylation assays were performed with saline and normal sera as negative controls. Briefly, 1x10^7 SKBR3 cells (human breast cancer cell line that overexpresses H2N) were harvested and incubated with human Ig (100 ug/ml) or c-neu-5 at 10 ug/ml for 2, 5, 10, and 15 minutes. At the end of incubation, cells were lysed with buffer containing sodium vanadate to inhibit further phosphorylation, and 1 mg of protein form each lysate from each incubation were immunoprecipitated with an anti-phosphotyrosine antibody. Immunoprecipitates were resolved on a 7.5% SDS gel and proteins transferred to nitrocellulose. Blots were probed with c-neu-3, a H2N specific monoclonal antibody to identify tyr-phosphorylated H2N. Identical blots were probed with an anti-phosphotyrosine antibody which recognizes a different epitope than the antibody used for immunoprecipitation. Panel A shows that the level of tyrosine phosphorylated H2N is decreased in the presence of patient sera recognizing ECD. The control H2N specific antibody c-neu-5 and patient sera with no H2N reactivity did not affect phosphorylation. Panel B demonstrates that other phosphorylated proteins, found at the same molecular weight, exhibited no changes in tyrosine phosphorylation. One interpretation is that human sera specific for the H2N ECD reduces phosphorylation of the H2N tyrosine kinase.

It is unknown whether changes in phosphorylation of the new tyrosine kinase translate into function effects. Soft colony agar formation assays to directly measure cell growth after incubation with neu specific human antibodies have been developed. Figure 5 shows growth inhibition of SKBR3 cells incubated with patient sera recognizing ECD and a control H2N ECD specific antibody, 7.16.4. The control antibody has been shown to bind and inhibit tumor growth both in vitro and in vivo [Katsumata et al. Nat Med 1:644, 1995]. To date, 10 patients have been identified whose H2N specific antibody response is ECD directed. Studies are ongoing to determine how many of these patients demonstrate functional ECD
antibodies.

Task 3: Months 1-48  To determine whether responses to H2N are beneficial or detrimental, stratifying for function.

These studies require sera from large cohorts of breast cancer patients. The H2N status needs to be known for all and all need to be treated with the same regimen. No such sera is available. 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. Large volume, well-defined, breast cancer clinical databases with long term follow up and corresponding serum samples are essential for answering many important questions about breast cancer etiology, prevention, diagnosis, and treatment and are sorely lacking at this time. We explored all of the major oncology cooperative groups as well as several cancer centers well known for large volume breast cancer therapy trials and were surprised at the lack of availability of banked sera on defined patients. We were not able to adequately determine the role of serum Ab to H2N in the evolution of malignancy and the correlation between extant immunity and outcome, because of a lack of available banked sera on well defined patients.

Therefore, to answer the questions in Aim #1 Task #3 we set up a serum bank for the National Surgical Adjuvant Breast and Bowel Project (NSABP). We began collecting sera as of the date of 3/1/97. The NSABP maintains a comprehensive clinical database on study participants, but has not previously collected sera, with the exception of their cancer prevention trial called P-01. Funds from the current grant are not going to fund the serum bank, but will be used to ask specific questions posed on the grant as to the role of H2N Ab on outcome. In addition, with the NSABP, we have initiated a study specifically designed to determine whether responses to H2N are beneficial or detrimental. A study of antibody responses to H2N 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 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 H2N 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 within our lab. 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.

To date we have collect initial sera on >50 individuals with H2N positive breast cancer. Serial serum is beginning to be collected on the same individuals. All patients are receiving the same adjuvant chemotherapy regimen. Thus, the variable of H2N reactivity can be analyzed. Stored sera will be examined in batches for Ab to whole H2N protein, ICD and ECD. The function of ECD reactive Ab will be determined. Circulating serum ECD levels will be measured. The level of H2N overexpression on primary tumor will be determined by immunocytochemistry on tissue blocks by NSABP reference pathologists. The NSABP statistical group will provide correlations.

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 H2N 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 H2N specific antibody response and survival, sufficient time points of sera will be collected that we may be able to discern whether H2N antibody levels vary predictably with recurrence. The patients included in the study above will have H2N protein overexpression assessed on their primary tumor as well as circulating levels of ECD, shed H2N protein, measured. Therefore, antibody data collected on these patients will allow the comparison of rise in antibody titer to the measurement of a more “classic” serum tumor marker; shed ECD protein.

In addition, we have begun to follow newly
diagnosed H2N positive breast cancer patients here at the University of Washington. Sera is obtained on the patients at the time of diagnosis and every 3 months for 3 years. To date 3 patients have been studied, 2 stage II patients and 1 stage III patient. More than 3 time points have been obtained on each patient. 1 patient, a stage II patient, had pre-existing H2N specific antibody and T cell response prior to starting chemotherapy. Within 3 months of treatment, while the antibody titer remains stable at 1:100, the H2N specific T cell response has become undetectable. The T cells, however, still respond to the non-specific mitogen, PHA. A longer period of time and more patients are needed to be enrolled on study to determine the natural history of H2N antibody and T cell levels with remission and recurrence of breast cancer.

Task 5: Months 13-48  To determine whether immunity to H2N: (a) is present in patients with DCIS; (b) correlates with progression of H2N+ DCIS to H2N negative invasive cancer; and (c) represents a possible marker for early cancer.

Some patients with breast cancer have both a preexistent antibody and CD4+ T helper cell immune response against H2N. In preliminary studies developing quantitative assays for CD4+ T cell responses, 62 breast cancer patients were tested. 11/62 (18%) had some level of H2N specific T cell response. 5/11 had substantial responses, with stimulation indices (SI) greater than 2. In the remainder of patients, no responses to H2N were observed. In 15 patients with no detectable response to H2N, no response to tetanus toxoid was detected, suggesting that at least some of these were immunoincompetent due to tumor burden or cancer-related treatments. Studies of breast cancer biopsies show that H2N levels are increased in the majority of DCIS specimens, but are not seen in atypia or dysplasia. Thus, overexpression of H2N appears to be associated with malignant transformation and early neoplasia, but not benign proliferative diseases of the breast. This observation raises the question whether H2N negative invasive ductal breast cancer arises from H2N positive DCIS and whether H2N immunity plays any role in immunoselection of progressive H2N negative invasive ductal cancer from H2N 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 H2N positive cancer.

In a collaboration with Dr. Ken Bertram, Madigan Army Hospital, Tacoma, WA, we collected sera and whole blood for the isolation of lymphocytes from 14 patients with DCIS. These patients had blood drawn within 2 years of the diagnosis of DCIS. 36% (5/14) had significant T cell responses directed toward the ECD protein (n=4) or the ICD protein (n=2), one patient had significant T cell immunity directed toward both recombinant domains. Epitope mapping of the T cell response with selected peptides in this patient revealed the T cell response was primarily directed towards two H2N 15 mer peptides, p328 (ECD) and p971 (ICD). Compared to our historical control population of breast cancer patients described above, with H2N specific T cell responses of 18% overall, it would appear that significant H2N specific T cell immunity was more prevalent in DCIS. The control population, however, consists of patients at all stages of disease and time of treatment. Studies are still underway analyzing responses in early stage H2N breast cancer patients evaluated at the time of diagnosis as a more appropriate control. H2N protein overexpression in DCIS is associated with comedo type rather than non-comedo type disease. Pathologic evaluation of the primary tumors in these patients was not available to make a correlation with H2N specific immunity and DCIS phenotype.

To address this question, collaboration has been made with Drs. Robin Ward and Nick Hawkins at St. Vincent’s Hospital and the University of New South Wales, Sydney, Australia. Over the next 8 months sera and cells on 30 patients with comedo type ductal carcinoma will be collected at the time of diagnosis and stored in liquid nitrogen. Blocks will be obtained for evaluation of H2N overexpression in the primary lesions. Samples will then be sent blinded for evaluation of H2N specific T cell and antibody immunity. In addition, 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 predate diagnosis. The prevalence of Ab to H2N will be too low to provide a general screening
assay for breast cancer. However, H2N is only one of many proteins involved in malignant transformation for which Ab immunity has been observed. Immunity has been detected to at least four common oncogene encoded proteins associated with breast cancer including, H2N, ras, p53 and c-myc. Results from studying H2N as a prototype should indicate whether the general approach will be fruitful.

Task 6: Months 1-48 To determine whether immunity to H2N correlates with outcome of 2B1 bispecific Ab therapy.

Preliminary data demonstrated that several patients treated with the 2B1 bispecific antibody developed antibody immunity to H2N. Experiments were proposed to determine whether any benefit accrued as the result of stimulation of human anti-H2N Ab responses. The clinical trials continue to be performed by Dr. Louis Weiner at the Fox Chase Cancer Institute. We have transferred the assay technology to Dr. Weiner's laboratory and his group will be conducting the proposed experiments.

We have also developed a collaboration with the group at Dartmouth, during the tenure of Dr. Frank Valone, evaluating H2N specific antibody immunity in patients who have received the bispecific antibody MDX-210. MDX-210 binds simultaneously to type I Fc receptors for immunoglobulin G (IgG) and to the H2N protein. Initial studies, of 10 patients treated with the murine bispecific construct, revealed levels of HAMA that obstructed the identification of any H2N specific antibody response. Despite aggressive "pre-clearing" protocols incubating the human sera with murine Ig, in only 30% of the patient sera were the anti-mouse antibodies cleared sufficiently to potentially detect H2N specific responses using the sandwich ELISA. Once recombinant proteins were constructed, samples were analyzed for antibody responses to H2N ICD and ECD. Serial sera were available on each patient, pre-treatment and at 3 time points post treatment. No pre-existing or developing H2N antibody responses were detected. MDX-210 was humanized and an additional 25 patients evaluated. Again, sera was available pre-treatment and at 3 time points following antibody infusion. Only two patients were found to have H2N specific antibodies; 1 patient did not change antibody titer through the course of treatment. The second patient increased H2N specific antibody titer from 1:50 pre-treatment to 1:100 post treatment. No patients developed H2N specific antibodies while being treated. Studies are ongoing with both groups to determine the H2N specific antibody response and the variability of eliciting H2N specific antibodies between the two bispecific constructs.

Specific Aim # 2: To examine CD4+ T cell immunity to HER-2/neu.

Task 7: Months 1-36 To develop in vitro priming with dendritic APC to generate H2N-specific CD4+ T cells and to identify the epitopes recognized.

Prior to developing priming methodology, it was necessary to define which portions of the H2N molecule are potentially immunogenic. The immunogenicity of self proteins, such as H2N, is dampened by immunological tolerance. Tolerance is considered to be directed primarily against immunodominant epitopes. Many potential epitopes are ignored. The ignored epitopes are termed "subdominant" epitopes. In animal studies, not funded by the current grant, we showed that peptide based vaccines in rats utilizing subdominant epitopes can be an effective way to overcome tolerance and to immunize to H2N. The rat experiments used rat neu protein, a molecule that is highly homologous to human H2N protein.

Potential subdominant H2N peptide epitopes were first selected based on motifs and in vitro evaluation. Peptides for class II MHC restricted CD4+ helper T cell responses were selected for study using a computer protein sequence analysis package of searching algorithms. The algorithms identified motifs for binding to class II MHC according to alpha helical orientation and to particular charge and polarity patterns and tertiary structure. Although peptides that bind to class II molecules are now known not to necessarily form alpha helical orientations, each of the searching algorithms had empirically been successful in identifying a substantial proportion (50-70%) of helper T cell epitopes in foreign proteins. Analysis resulted in the identification of more than 40 H2N peptides with the potential to interact with human class II MHC.

Based on predicted interactions, 26 peptides, 15-18 amino acids in length were constructed. Heparinized blood was obtained from over 60 breast cancer patients and lymphocytes isolated and tested for proliferative response in standard and modified limiting dilution analysis based on tritiated thymidine incorporation. Immune
response to seven of 26 peptides tested were detected in vitro in at least 20% of breast cancer patients evaluated. Thus, extensive empirical testing of proposed helper epitopes in vitro resulted in the identification of potential subdominant epitopes of H2N. These peptides are being used in studies to prime in vitro.

Studies have focused on priming in vitro as a possible rapid and inexpensive way to identify immunogenic epitopes for eventual inclusion into peptide based vaccines. Initial studies generated and used autologous, bone marrow-derived dendritic cells (DC) as antigen presenting cells (APC) to rapidly generate antigen specific CD4+ T cells from the peripheral blood lymphocytes (PBL) of normal individuals. Those studies showed that functional dendritic cells could be elicited and grown in vitro from CD34+ HPC derived from bone marrow or G-CSF mobilized peripheral blood. Culture of CD34+ HPC with GM-CSF and TNF-α yielded a heterogeneous cell population containing cells with typical dendritic morphology. Phenotypic studies demonstrated a loss of the CD34 molecule over one week and an increase in cells expressing surface markers associated with dendritic cells, CD1a, CD80 (B7/BB1), CD4, CD14, HLA-DR, and CD64 (FcγRI). Function was validated in experiments showing that cultured cells could stimulate proliferation of allogeneic CD4+ and CD8+ T lymphocytes. Antigen presenting capacity was further confirmed in experiments showing that cultured cells could effectively stimulate H2N peptide specific responses.

Having shown that bone-marrow-derived DC can be used for priming human T cells, we then compared the efficacy of bone-marrow-derived DC with blood-derived DC for priming human T cells, since blood is more easily obtained from human subjects than is bone marrow. We found the blood-derived DC to be equally effective in the stimulation of antigen specific T cell responses, and have begun to use these APC. This has increased our donor pool and eliminated the need for procuring bone marrow.

We initially worked out priming conditions using foreign antigens including KLH, ovalbumin, hen egg lysozyme, and beta-galactosidase. Using either bone marrow- or blood-derived DC, we have been able to prime naive (CD45RA+) CD4+ T cells to the prototype antigens. Specificity has been validated by showing that DC-stimulated CD4+ T cells can respond to the priming antigen, but not to an irrelevant antigen, when tested following restimulation and expansion. Now we are applying the systems to H2N.

We have used both H2N peptides and recombinant proteins. The extracellular and intracellular domains of the H2N protein were synthesized and purified from an E. coli expression system for use in these studies. Our findings with the E. coli derived protein is that CD4+ T cell proliferative responses can be elicited to the H2N protein preparations; however, it has proven difficult to demonstrate the specificity of the T cells, most likely due to low level bacterial-derived contaminants in the protein preparations. We are currently attempting to improve the quality of the proteins by expressing them in mammalian cells. Preliminary results using the extracellular domain of the H2N protein synthesized in this mammalian system are promising, and we plan to continue our efforts to elicit CD4+ cells specific for H2N using these more highly purified reagents.

Task 8: Months 13-48 To determine whether Th immunity to H2N can be generated using T cells from individuals with H2N+ breast cancer and no detectable immunity to H2N.

Tasks 8 will be embarked upon this year after further development of systems for priming, as presented above in task 7.

Task 9: Months 13-48 To determine the prevalence of CD4+ responses in patients with H2N+ tumors and the evolution of immunity with therapy and relapse.

This task requires serial assays. Assessment of serial assays has required the development of assays that are quantitative and statistically based. The challenge is designing quantitative assays while using only a limited amount of patient material. In the trial, H2N specific T cell proliferation is assessed by using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors. This method is based on Poisson distribution, a more appropriate method for identifying randomly distributed responding cells of low number in replicate wells. A cutoff value of the mean and 3 standard deviations of the no antigen wells is used to score a well as responding or non-responding. All antigens are set up in 24 well replicates. Responses can be quantified as the number of responding wells of 24 well replicate. Using this cutoff value, one has a 95% confidence interval
that a well is positive due to the specific antigen added. This assay system allows for quantification of H2N specific T helper responses after each sequential immunization and use a limited number of cells. Similar quantitative statistically based assays have been developed for the assessment of H2N specific CTL responses. The modified LDA will now be used to determine the prevalence of CD4+ responses in patients with H2N+ tumors and the evolution of immunity with therapy and relapse.

Task 10: Months 13-48  To determine whether CD4+ responses modulate the biology of autologous tumors in vivo.

The frequency of Ab to H2N was determined in experiments described in Task #1 with well defined sera provided by the Milano group. The studies were possible because stored sera was available. PBL are not available on similar retrospective series of patients. Thus, we have begun to follow a group of patients with H2N+ cancers. PBL are being collected at the time of diagnosis and at 3 month intervals during the course of treatment, chemotherapy, radiation therapy, and then at 6 month intervals once treatment has finished to 2-5 years after treatment. As detailed in Task 4, we have begun blood collection on 3 individuals with H2N positive cancers. More will be added. By following a group of patients, we are attempting to compare the clinical course of patients with H2N+ cancer who have detectable responses to H2N to patients who do not have detectable immune responses.

Specific Aim # 3: To examine CD8+ CTL immunity to HER-2/neu.

Task 11: Months 1-48  To develop in vitro priming with dendritic APC to generate H2N specific CD8+ T cells and to identify the epitopes recognized.

We have embarked on studies to determine the most efficient, physiologically relevant, means of generating H2N-specific CTL by priming in vitro. Several approaches are being studied including experiments 1) to elicit CTL by in vitro priming with peptides and dendritic cells (DC), 2) to elicit CTL by in vitro priming with DC transfected with either DNA or RNA encoding H2N, and 3) to elicit CTL by in vitro priming with DC infected with recombinant virus encoding H2N. Each method has distinct advantages and disadvantages. Each has yielding encouraging results. None have solved the essential question as to how best to prime and how to prime reproducibly.

We already know that priming CD8+ T cells with peptides and DC will elicit peptide-specific CTL. However, the systems are not yet reproducible and the drawback is that not many of the peptide-specific CTL also recognize cells expressing the native protein. We will continue some of our efforts using this strategy, because it works in some cases. One problem is that use of high concentrations of peptide may yield T cells most adept at recognizing unphysiologic concentrations of peptide above that normally expressed by cancer cells. Thus, efforts will be directed to priming with lower concentrations of peptides.

Additional efforts will be focused on generating CTL responses to APC expressing the entire protein. This may allow priming to more physiologic concentrations of naturally processed peptides. To this end, we plan to optimize transfection of DC with DNA and/or RNA so that sufficient numbers of cells and levels of protein are produced to allow T cell recognition. We have constructed the vectors needed for these experiments, and have shown that they are functional in murine cell lines that are more easily transfected. Nucleic acid transfection of DC would be the preferred method for getting the protein of interest into the class I pathway because it does not involve expression of any other foreign proteins and, thus, should improve the probability of priming to the protein of interest.

An alternative method of expressing H2N for priming that we have tested is by expressing it as a recombinant protein by vaccinia virus. This was in effective, in that we were able to prime in vitro to the construct. However, by far and away the dominant response was to vaccinia virus encoded antigens. In some experiments we could detect small numbers of H2N specific CTL, but they were rare and not present in all cases. To overcome the dominance of vaccinia virus encoded antigens, we plan to repeat this experiment using vaccinia-H2N-infected DC in the priming and autologous APC transfected with H2N in a vaccinia-negative vector restimulations. This may eliminate the expansion of vaccinia virus-reactive T cells. Such a vector containing the entire H2N cDNA is available in the lab and has already been used to successfully transfect donor B cells.

Task 12: Months 1-36  To determine whether CD8+ CTL immunity can be generated using T
cells from individuals with H2N+ breast cancer and no detectable immunity to H2N.

Studies to date to learn how to prime in vitro have focused on T cells from normal individuals. Experiments in Task #12 will await optimization of priming as presented above in Task #11.

Task 13: Months 37-48 To determine the prevalence of CD8+ responses in patients with H2N+ tumors and the evolution of immunity with therapy and relapse.

In order to perform these experiments it has been necessary to develop a highly reproducible assay system for quantifying CTL. We have developed a modified LDA for CTL similar to the assay described in Task 9 for quantifying helper T cell responses. This method is also based on Poisson distribution of positive and negative wells. PBMC are stimulated in 24 well plates and examined for lytic ability against H2N peptides. A cutoff value of the mean and 3 standard deviations of the no antigen wells is used to score a well as responding or non-responding. The modified LDA will now be used to determine the prevalence of CD8+ responses in patients with H2N+ tumors and the evolution of immunity with therapy and relapse. We have begun to store frozen PBL on patients with H2N+ cancers, but have not yet begun analysis patient responses.

Task 14: Months 13-48 To determine whether H2N-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.

The major problem with evaluation of responses to autologous tumor is a lack of availability of autologous tumors. We have therefore initiated a collaborative effort with E. Repasky at RPMI. Dr. Repasky has been able to implant primary breast cancers into SCID mice. Thus, far she has been able to implant and have grow >50 primary breast cancers. Eventually, Dr. Repasky will provide autologous tumor and PBL to perform the proposed experiments. Given that she now as a collection of H2N positive autologous cancers, the studies will be possible.

We are currently performing experiments to determine whether H2N specific T cells can be expanded and the extent to which such T cells can be expanded. The culture conditions being evaluated include the use of serum, type of serum, exogenous cytokines and form of antigen.
CONCLUSIONS:

The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against H2N. The studies thus far have validated that immunity to H2N exists and can be augmented by manipulation in vitro. The studies have thus far provided great assistance in developing H2N vaccines.

Studies of antibody responses confirmed that Ab immunity to H2N can be detected in the sera of some patients with breast cancer. The increased frequency of Ab in patients with H2N+ cancer strongly implies that immunity develops as the result of the overexpression of H2N on breast cancer, i.e., some patients become immune to their own cancers. The confirmation that some patients become immune to their own cancers gives great credence to the concept that vaccines will be able to elicit substantial immunity to H2N.

H2N is a functioning growth factor receptor. Ab to H2N in some patients was directed against the ECD and was able to perturbate function. This remarkable finding strongly implies that the immune response to H2N might directly alter growth characteristics and outcome in patients with breast cancer. Whether patients with H2N+ cancers and functional Ab to H2N survive longer as a result of Ab is being pursued. 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 H2N relates to improved survival is being addressed using sera collected from the NSABP adjuvant breast cancer trials. Sera is drawn at the time of diagnosis and all patients are receiving the same adjuvant chemotherapy regimen. Thus, H2N reactivity can be analyzed as an independent variable.

Studies of CD4+ T cell immunity to H2N confirmed that some patients with H2N-positive breast cancers exhibit primed CD4+ helper T cell responses to H2N. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy given that H2N 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.

For vaccine development identification of peptide epitopes is important. Extensive empirical testing of putative helper T cell epitopes in vitro resulted in the identification of potential vaccine candidate epitopes. Some of these peptides have been incorporated into a peptide based vaccine that we are currently testing in Phase I clinical trials funded by a different mechanism. To facilitate the search for additional peptide epitopes, we have developed the use of blood-derived dendritic cells to stimulate antigen specific T cell responses. This has increased the ease of priming in vitro.

We have developed highly sensitive and reproducible modified limiting dilution assays for evaluating CD4+ and CD8+ T cell responses to H2N. This assays are being used in the current studies to identify, quantify and follow existent immune responses to H2N. In addition, the assays have proven to be of great value for quantifying responses in a H2N peptide based vaccine trial that we have recently initiated.

Studies of CD8+ T cell immunity to H2N confirmed that CD8+ CTL can be primed to H2N peptides in vitro and that primed peptide specific CTL can lyse H2N positive cancer cells. We have embarked on studies to determine the most efficient, physiologically relevant, means of generating H2N-specific CTL by priming in vitro including in vitro priming with peptides, dendritic cells transfected with either DNA or RNA and dendritic cells infected with recombinant virus encoding H2N. Each method has yielded encouraging results, but with different advantages and disadvantages. No method has solved the essential question of how best to prime and how to prime reproducibly.

A major issue is whether H2N-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor. To accomplish this goal, we now have been able to establish multiple autologous breast cancers growing in SCID mice that will be used as a source of autologous tumor.

Finally, we have begun the experiments necessary to determine whether H2N specific T cells can be expanded and whether such cells can be expanded with maintenance of function to the extent presumed necessary for therapy.
Figure 1. Stage III and IV breast cancer patients have significant antibody responses to dermatophytin. Antibody responses to dermatophytin were assessed in advanced stage patients as a measure of immunocompetence. Sera from the 32 advanced stage breast cancer patients and 40 sera from normal blood donors were analyzed by ELISA for antibodies to dermatophytin. Sera from an individual with high titer dermatophytin specific antibodies was used as a positive control. The data is expressed as the mean and standard deviation of the experimental O.D. of each population. The standard deviation of the positive control between assays was less than 0.02 O.D.

Figure 2. H2N specific antibodies can be quantitated in patient serum samples. The 3 closed circles represent breast cancer patients, AS, VB and LB who were analyzed for H2N antibodies using a quantitative sandwich ELISA. These patients were previously shown to have H2N specific antibody titers of 1:200 (AS) and 1:100 (VB, LB). Each sample was analyzed in quadruplicate. The value shown is the mean and standard deviation of these 4 values. The shaded circle represents values from 50 normal blood donors. Open crosses are the values derived from purified human IgG which comprise the standard curve.

Figure 3. Immune sera from patients with breast cancer can be used to detect novel immunogenic proteins. Sera from 9 cancer patients and 1 normal control were used as primary antibody in an immunoblot using lysates of SKBR3. x indicates patients with known H2N specific antibodies (positive control). The arrows demonstrate some novel immunogenic tumor cell proteins detected by breast cancer patient, but not normal sera.
Figure 4. Sera from breast cancer patients with ECD antibody response can effect the phosphorylation of HER-2/neu protein. Sera was obtained from a patient with a H2N ECD and p328 specific antibody response. Ig was purified from the sera by separation over an agarose A column. c-neu-5 is a H2N ECD specific monoclonal antibody that has been shown to increase phosphorylation of neu tyrosine kinase when it is incubated with H2N positive tumor cells. Phosphorylation assays were performed with the purified human Ig, c-neu-5 as a positive control, and saline, as well as normal sera, as negative controls. All lysates were quantitated for protein concentration prior to analysis and 10 µg of total protein was loaded in each lane. Results shown here depict incubations of 10 minutes. Sera specific for the H2N ECD reduces the phosphorylation of the H2N tyrosine kinase.

Figure 5. Human HER-2/neu ECD specific antibody can inhibit the growth of SKBR3 cells in culture. Two layer soft agar assays were used to evaluate anchorage independent growth of SKBR3. Briefly, 0.6% agar in tissue culture medium (TCM) was solidified as the basal layer in 35 mm tissue culture dishes. 5x10^3 SKBR3 were suspended in 1 ml 0.3% agar in TCM alone or in TCM supplemented with 7.16.4 (10ug/ml) or human purified Ig (100 ug/ml). The 7.16.4 antibody has been shown to inhibit the growth of breast cancer cells in vivo, and was used as a positive control. The cells were preincubated for 10 minutes in TCM mixed with appropriate antibodies and then mixed with the agar. Plates were incubated for 10 days and then colonies of SKBR3 were counted by light microscopy. A group of > 20 cells is considered a colony. Data is expressed as the mean and standard deviation of quadruplicate cultures.