Vaccination against the HER-2/neu oncogenic protein

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Abstract

The HER-2/neu oncogenic protein is a well-defined tumor antigen. HER-2/neu is a shared antigen among multiple tumor types. Patients with HER-2/neu protein-overexpressing breast, ovarian, non-small cell lung, colon, and prostate cancers have been shown to have a pre-existent immune response to HER-2/neu. No matter what the tumor type, endogenous immunity to HER-2/neu detected in cancer patients demonstrates two predominant characteristics. First, HER-2/neu-specific immune responses are found in only a minority of patients whose tumors overexpress HER-2/neu. Secondly, immunity, if detectable, is of low magnitude. These observations have led to the development of vaccine strategies designed to boost HER-2/neu immunity in a majority of patients. HER-2/neu is a non-mutated self-protein, therefore vaccines must be developed based on immunologic principles focused on circumventing tolerance, a primary mechanism of tumor immune escape. HER-2/neu-specific vaccines have been tested in human clinical trials. Early results demonstrate that significant levels of HER-2/neu immunity can be generated with active immunization. The T-cell immunity elicited is durable after vaccinations have ended. Furthermore, despite the generation of CD8+ and CD4+ T-cells responsive to HER-2/neu in a majority of patients, there is no evidence of autoimmunity directed against tissues that express basal levels of the protein. Cancer vaccines targeting the HER-2/neu oncogenic protein may be useful adjuvants to standard therapy and aid in the prevention of relapse in patients whose tumors overexpress the protein. Furthermore, boosting HER-2/neu-specific T-cell frequencies via active immunization may allow the ex vivo expansion of HER-2/neu-specific T-cells for use in adoptive immunotherapy, a therapeutic strategy directed against the treatment of established disease.

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Introduction

The HER-2/neu protein consists of a cysteine-rich extracellular ligand binding domain, a short transmembrane domain, and a cytoplasmic protein tyrosine kinase domain (Samanta et al. 1994, Olayioye et al. 2000). Binding of ligand to the extracellular domain (ECD) leads to dimerization that stimulates the intrinsic tyrosine kinase activity of the receptor and triggers autophosphorylation of specific tyrosine residues within the intracellular cytoplasmic domain (ICD). These phosphorylated residues then serve as anchoring sites for signaling molecules involved in the regulation of intracellular signaling cascades (Olayioye et al. 2000) and, thus, cell growth.

HER-2/neu is a self-protein expressed in a variety of tissues of epithelial origin and it plays a fundamental role in cellular proliferation and differentiation during fetal development. In adults, the HER-2/neu gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression is seen in various cancers including breast, ovarian, colon, uterine, gastric, prostate, and adenocarcinoma of the lung. Furthermore, the overexpression of HER-2/neu is implicated in the malignant transformation of breast cancer (Allred et al. 1992, Stark et al. 2000, Allred et al. 2001) and is a biologically relevant protein in the pathogenesis of several other epithelial-based tumors, for example leading to the development of hormone resistance in prostate cancer (Craft et al. 1999).
Generating an active immune response directed against the HER-2/neu protein has several potential clinical advantages. Vaccination, if effective, would stimulate immunologic memory and could result in the prevention of relapse after standard therapy such as surgery and radiation had been administered. Relapse in patients with breast and ovarian cancer, in a high-risk category due to HER-2/neu protein overexpression, is a major clinical problem (Slamon et al. 1987). In addition, if antibody immunity could be generated by active immunization, durable levels of functional antibodies binding the ECD of the growth factor receptor could be elicited if appropriate epitopes were targeted. Compelling evidence that the HER-2/neu protein may be a reasonable vaccine candidate is the observation that patients with HER-2/neu overexpressing tumors have low level pre-existent immunity directed against the protein. Thus, over the past decade immunologic investigations focusing on the HER-2/neu protein have progressed from pre-clinical studies, defining HER-2/neu as a tumor antigen and using the pre-existent immune response to HER-2/neu present in cancer patients to develop vaccines, to clinical studies actively immunizing cancer patients against HER-2/neu and developing strategies to use HER-2/neu T-cell immunity as a treatment for established tumors.

The HER-2/neu oncogenic protein is a tumor antigen

Cancer patients have pre-existent immunity to HER-2/neu

Patients with a variety of cancers whose tumors overexpress HER-2/neu can have pre-existent antibody and T-cell immunity directed against the antigen. In general, immunity to HER-2/neu in cancer patients is of low magnitude and found only in a minority of patients with HER-2/neu-overexpressing tumors. Of note, cancer patients with pre-existing antibody or T-cell immunity to HER-2/neu show no evidence of autoimmune disease, suggesting that antibodies and antigen-specific T-cells that arise in association with overexpression of the oncogenic protein do not recognize cells expressing basal levels of HER-2/neu. Furthermore, immunity to HER-2/neu can be found in a variety of tumors, underscoring HER-2/neu as a shared tumor antigen in multiple different tissue types.

Antibody immunity directed against the HER-2/neu protein has been most widely studied. Investigations of HER-2/neu-specific antibodies in patients with breast cancer demonstrate that responses can be detected in patients with early stage disease, indicating that the presence of antibodies is not simply a reflection of tumor burden. HER-2/neu antibodies of titers $>$ 1:1000 were detected in 12 of 107 (11%) breast cancer patients compared with 0 of 200 (0%) controls (Disis et al. 1997). Detection of antibodies to HER-2/neu also correlated with protein overexpression in the patient’s primary tumor. A subsequent study evaluated 45 patients with advanced stage (III/IV) HER-2/neu-overexpressing breast and ovarian cancer for detection of pre-existent humoral immunity (Disis et al. 2000). Only 7% had detectable HER-2/neu-specific IgG antibodies tumors. HER-2/neu protein overexpression is detected in 30–50% of colon cancers (Ward 1999). Antibodies to HER-2/neu have been found in the sera of patients with colon cancer; titers of $>$ 1:1000 were detected in 8 of 57 (14%) patients with colorectal cancer compared with 0 of 200 (0%) of the normal control population. Similar to the immune response in breast cancer patients, the ability to detect HER-2/neu antibodies correlated with overexpression of the protein in the patient’s primary tumor (Ward 1999). Finally, HER-2/neu antibody immunity has been studied in prostate cancer. Detection of HER-2/neu-specific antibodies was significantly higher in patients with prostate cancer (15.5%, 31 of 200) compared with controls (2%, 2 of 100), and titers $>$ 1:100 were most prevalent in the subgroup of patients with androgen-independent disease (McNeel et al. 2000).

Existing T-cell immunity to the oncogenic protein, both T-helper and cytotoxic T-cells (CTL), have been detected in patients with HER-2/neu overexpressing tumors. The identification of T-cells that can respond to HER-2/neu indicates that a portion of the T-cell repertoire will recognize this self-antigen. Furthermore, it may be more appropriate, when developing vaccine strategies designed to circumvent tolerance, to immunize patients to boost weak pre-existent responses rather than prime a de novo HER-2/neu-specific immune response in patients. Both CD4$^+$ and CD8$^+$ T-cell responses were evaluated in patients with advanced stage HER-2/neu-positive tumors (Disis et al. 2000). These patients had not received immunosuppressive chemotherapy for at least 30 days (median 5 months, range 1–75 months) prior to entry in the study. All patients were documented to be immunocompetent by delayed type hypersensitivity (DTH) testing using a skin anergy battery. Five of the 45 patients (11%) were found to have a detectable HER-2/neu protein-specific T-cell response as defined by a stimulation index $>$ 2.0 (range 2.0–7.9). A limited number of patients were human leukocyte antigen (HLA)-A2-positive and were evaluated for CD8$^+$ T-cell immunity to a dominant HLA-A2 epitope derived from the HER-2/neu ECD, p369–377 (Fisk et al. 1995). None of the 8 patients evaluated had a precursor frequency $>$ 1:100 000 peripheral blood mononuclear cells (PBMC) to p369–377. However, 5 of 7 patients had significant levels of flu-specific immunity (mean 1:20 312, range 1:31 250–1:13 700) demonstrating anergy was not responsible for the lack of CD8$^+$ response to the tumor antigen. Cytotoxic T-cells capable of lysing HER-2/neu-overexpressing tumor cell lines have been identified in both the peripheral blood and tumors of patients bearing a variety of HER-2/neu-overexpressing tumors. Early studies identified HER-2/neu-
specific CTL in the malignant ascites of HLA-A2-positive patients with HER-2/neu-overexpressing ovarian cancer (Ioannides et al. 1993). Similar investigations have isolated tumor-specific CTL from tumor infiltrating lymphocytes of HLA-A2-positive HER-2/neu-overexpressing non-small-cell lung cancer (NSCLC). These CTLs specifically recognized HLA-2+HER-2/neu+ autologous and allogeneic NSCLC cell lines as well as HLA-matched and antigen-positive ovarian cancer cell lines (Yoshino et al. 1994). In addition, studies have identified HER-2/neu-specific CTL in patients with HER-2/neu-overexpressing breast, ovarian, renal cell, pancreatic, gastric, colon and lung cancers (Yoshino et al. 1994, Peoples et al. 1995, Brossart et al. 1998, Kono et al. 1998, Peiper et al. 1999). HER-2/neu-specific T-cells, isolated from cancer patients, can aid in the identification of epitopes appropriate for inclusion in vaccines.

HER-2/neu vaccine development focuses on strategies that will allow tolerance to be ‘circumvented’

The development of peptide-based vaccines may be uniquely suited to stimulate immunity to a self-antigen such as HER-2/neu. The ability to mount an immune response is related to the immunodominance of specific antigenic determinants during natural immunologic processing of intact protein antigens. However, only a minor fraction of potential determinants in an antigen are presented in an immunodominant manner, while the remaining peptides are ignored (Sercarz et al. 1993). Usually, physiological mechanisms of immunologic tolerance to self prevent the induction of an immune response to self-proteins, such as HER-2/neu. Dominantly processed self-determinants are thought to be efficient in tolerance induction (Sercarz et al. 1993, Nanda & Sercarz 1995). However, in every self-antigen, there are sequestered determinants that are unable to induce tolerance and therefore could be immunogenic (Sercarz et al. 1993). These subdominant epitopes may trigger the threshold for T-cell activation and immune recognition if they are presented in abundance, such as when a self-protein becomes overexpressed. Overexpression of the HER-2/neu protein may result in subdominant peptides being presented in higher concentration in the major histocompatibility complex (MHC), thus triggering a T-cell response. Potentially, the processed peptide repertoire in MHC could be distinctly different in a tumor cell where a self-protein was overexpressed than in a non-malignant cell where a self-protein is present at basal levels. Abundance of subdominant epitopes in MHC molecules expressed on cancer cells could result in overexpressed self-proteins functioning as tumor-specific antigens. An alternative hypothesis is that subdominant epitopes are more effectively presented by highly activated and efficient antigen presenting cells (APC), such as dendritic cells (DC), or APC markedly activated by inflammatory signals from the local immune microenvironment (Nanda & Sercarz 1995).

Computer modeling programs have been effective in predicting potential immunogenic epitopes of self-proteins such as HER-2/neu, and early studies have focused on evaluating constructed peptides for signs of immune reactivity in patients with HER-2/neu-positive tumors (Disis & Cheever 1998). MHC class I-binding epitopes can be identified and corresponding synthetic peptides tested for their capacity to induce peptide- and tumor-specific CTL derived from healthy individuals or cancer patients (Rongcun et al. 1999). Using this method, Rongcun and colleagues identified four HER-2/neu-specific HLA-A2.1 restricted CTL epitopes: HER2(9369), HER2(9435), and HER2(9689) which were able to elicit CTL that specifically killed peptide-sensitized target cells, and most, importantly, a HER-2/neu-transfected cell line and autologous tumor cells. In addition, CTL clones specific for HER2(9369), HER2(9435), and HER2(9689) epitopes were isolated from tumor-specific CTL lines, further demonstrating the immunogenicity of these epitopes. A similar strategy involves defining candidate epitopes by their MHC-binding motif and class I affinity (Keogh et al. 2001). Identified high affinity peptides are then tested for in vitro reactivity with PBMC from normal donors and the ability to induce tumor-reactive CTLs. A potential problem in the development of CTL epitope-based vaccines is the large degree of MHC polymorphism. However, it is now known that HLA class I molecules can be divided into several families or supertypes based on similar peptide-binding repertoires (Keogh et al. 2001). For example, the A2 supertype consists of at least eight related molecules, and of these the most frequently observed are HLA-A*0201, A*0202, A*0203, A*0206, and A*6802. In addition, the A2 supertype is expressed in all major ethnicities — in the 39–46% range of most common populations. Many peptides that bind A*0201 also exhibit degenerate binding (binding to multiple alleles); thus an A2 supertype multi-epitope vaccine could be designed to provide broad population coverage (Keogh et al. 2001).

The relationship between class I affinity and tumor antigen epitope immunogenicity is of importance because tissue-specific and developmental tumor antigens, such as HER-2/neu, are expressed on normal tissues at some point in time at some location within the body. T-cells specific for these self-antigens could be functionally inactivated by T-cell tolerance; however, several studies have now shown CTL responses to tumor epitopes in both normal donors and cancer patients, indicating that tolerance to these tumor antigens, if it exists at all, is incomplete (Kawashima et al. 1998, Keogh et al. 2001). Whether or not T-cells recognizing high-affinity epitopes have been selectively eliminated, leaving a repertoire capable of recognizing only low-affinity epitopes, is not known. Further studies evaluated several peptides derived from four different tumor antigens, p53, HER-2/neu,
correlated with high HLA-A2.1 binding affinity (IC50 revealed that recognition of naturally processed antigen was matched antigen-bearing cancer cell lines. Further analysis cells generated by 13/20 of the wild-type epitopes and 6/9 of and monocyte-derived dendritic cells as APC. Cytotoxic T- some antibodies have no effect on cell growth while others neu-specific antibodies inhibit the growth of cancer cells, breast cancer (Vogel demonstrated clinical efficacy in the treatment of metastatic been isolated and one such antibody, trastuzumab, has dem- tion and screening of HER-2/neu-specific peptide epitopes may aid the development of clinical vaccines for use in the treatment of HER-2/neu overexpressing tumors.

Another aspect of peptide epitope prediction would be to identify peptide portions of the HER-2/neu ECD that would be appropriate to target with an antibody response. Several monoclonal antibodies against the HER-2/neu ECD have been isolated and one such antibody, trastuzumab, has demonstrated clinical efficacy in the treatment of metastatic breast cancer (Vogel et al. 2001). Although many HER-2/ neu-specific antibodies inhibit the growth of cancer cells, some antibodies have no effect on cell growth while others even actively stimulate cancer growth (Yip et al. 2001). This wide range of biological effects is thought to be related to the epitope specificity of the antibodies and to consequent changes in receptor signaling (Yip et al. 2001). An alternative to the use of passive antibody therapy would be active immunization against the HER-2/neu ECD. However, inappropriately induced immune responses could have untoward effects on cancer growth. Therefore, it is crucial to identify epitopes on HER-2/neu that are targeted by stimulatory and inhibitory antibodies in order to ensure the induction of a beneficial endogenous antibody response.

In a recent study, investigators constructed HER-2/neu gene fragment phage display libraries to epitope-map a number of HER-2/neu-specific antibodies with different biological effects on tumor cell growth (Yip et al. 2001). Regions responsible for opposing effects of antibodies were identified and then used to immunize mice. The epitopes of three antibodies, N12, N28, and L87 were successfully located to peptide epitope binding regions of HER-2/neu. While N12 inhibited tumor cell proliferation, N28 stimulated the proliferation of a subset of breast cancer cell lines overexpressing HER-2/neu. The peptide region recognized by N12 was used as an immunogen to selectively induce an inhibitory immune response in mice. Mice immunized with the peptide developed antibodies that recognized both the peptide and native HER-2/neu. More importantly, HER-2/neu-specific antibodies purified from mouse sera were able to inhibit up to 85% of tumor cell proliferation in vitro. This study provides direct evidence of the function–epitope relationship of HER-2/neu-specific antibodies generated by active immunization. Using peptide regions that contain multiple inhibitory B cell epitopes is likely to be superior to the use of single epitope immunogens (Dakappagari et al. 2000). Current clinical trials of HER-2/neu vaccines largely focus on the use of peptide epitopes as immunizing antigens.

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carcinoembryonic antigen (CEA), and MAGE proteins, for their capacity to induce CTL in vitro capable of recognizing tumor target lines (Keogh et al. 2001). In order to increase the likelihood of overcoming tolerance, fixed anchor analogs that demonstrate improved HLA-A*0201 affinity and binding were used. Forty-two wild-type and analog peptides were screened. All the peptides bound HLA-A*0201 and two or more additional A2 supertypes alleles with an IC50 of 500 nM or less. A total of 20/22 wild-type and 9/12 single amino acid substitution analogs were found to be immunogenic in primary in vitro CTL induction assays, using normal PBMCs and monocyte-derived dendritic cells as APC. Cytotoxic T-cells generated by 13/20 of the wild-type epitopes and 6/9 of the single substitution analogs tested recognized HLA-matched antigen-bearing cancer cell lines. Further analysis revealed that recognition of naturally processed antigen was correlated with high HLA-A2.1 binding affinity (IC50 = 200 nM or less; P = 0.008), suggesting that high binding affinity epitopes are frequently generated and can be recognized as a result of natural antigen processing. Studies such as these demonstrate that recognition of self-tumor antigens is within the realm of the T-cell repertoire and that binding affinity may be an important criterion for epitope selection. Peptide-based vaccines have been found to be a strategy that will allow tolerance to be circumvented in animal models of neu immunization (Disis et al. 1996b). Therefore, rapid prediction and screening of HER-2/neu-specific peptide epitopes may aid the development of clinical vaccines for use in the treatment of HER-2/neu overexpressing tumors.

Human clinical trials of vaccines targeting the HER-2/neu oncogenic protein

Stimulating a cytotoxic T-cell response to HER-2/neu in vivo

The cytotoxic T-cell has been considered the primary effector cell of the immune system capable of eliciting an anti-tumor response. The predominant experimental method of stimulating a CTL response in vivo has been to vaccinate individuals with tumor cells or viruses recombinant for tumor antigens that can infect viable cells, so that proteins are exposed inside the cell and are processed and presented in the class I MHC antigen processing pathway. An alternative effective vaccination strategy to elicit CTL uses a soluble peptide that is identical or similar to naturally processed peptides that are present in class I MHC molecules along with adjuvant. An HLA-A2 binding peptide, p369–377, derived from the protein sequence of HER-2/neu ECD has been used extensively in clinical trials to generate CTL specific for cells overexpressing HER-2/neu in vivo via active immunization.

In an initial clinical study, HLA-A2-positive patients with metastatic HER-2/neu-overexpressing breast, ovarian, or colorectal carcinomas were immunized with 1 mg p369–377 admixed in incomplete Freund’s adjuvant (IFA) every 3 weeks (Zaks & Rosenberg 1998). Peptide-specific CTL were isolated and expanded from the peripheral blood of patients after 2 or 4 immunizations. The CTL could lyse HLA-matched, peptide-pulsed, target cells but could not lyse HLA-matched tumors expressing the HER-2/neu protein. Even
When tumors were treated with interferon γ (IFNγ) to upregulate class I, the CTL lines generated from the patients would not respond to the peptide presented endogenously on tumor cells. An additional problem in using single HLA binding epitopes is that, without CD4+ T-cell help, responses generated are short-lived and non-durable. More recently, a similar study was performed, immunizing patients with p369–377 using granulocyte macrophage colony-stimulating factor (GM-CSF) as an adjuvant (Knutson et al. 2002). GM-CSF is a recruitment and maturation factor for skin DC, Langerhans cells (LC) and, theoretically, may allow more efficient presentation of peptide epitopes than standard adjuvants such as IFA. Six HLA-A2 patients with HER-2/neu-overexpressing cancers received 6 monthly vaccinations with 500 µg HER-2/neu peptide p369–377, admixed with 100 µg GM-CSF. The patients had either stage III or stage IV breast or ovarian cancer. Immune responses to the p369–377 were examined using an IFNγ ELISPOT assay. Prior to vaccination, the median precursor frequency, defined as precursors/10⁶ PBMC, to p369–377 was not detectable. Following vaccination, HER-2/neu peptide-specific precursors developed to p369–377 in just 2 of 4 evaluable subjects. The responses were short-lived and not detectable at 5 months after the final vaccination. Immunocompetence was evident as patients had detectable T-cell responses to tetanus toxoid and influenza. These results demonstrate that HER-2/neu MHC class I epitopes can induce HER-2/neu peptide-specific IFNγ-producing CD8+ T-cells. However, the magnitude of the responses was low as well as short-lived. Theoretically, the addition of CD4+ T-cell helper epitopes would allow the generation of lasting immunity.

A successful vaccine strategy in generating peptide-specific CTL capable of lysing tumor expressing HER-2/neu and resulting in durable immunity involved immunizing patients with putative T-helper epitopes of HER-2/neu which had, embedded in the natural sequence, HLA-A2 binding motifs of HER-2/neu. Thus, both CD4+ T-cell helper epitopes and CD8+ specific epitopes were encompassed in the same vaccine. In this trial, 19 HLA-A2 patients with HER-2/neu-overexpressing cancers received a vaccine preparation consisting of putative HER-2/neu helper peptides (Knutson et al. 2001). Contained within these sequences were the HLA-A2 binding motifs. Patients developed both HER-2/neu-specific CD4+ and CD8+ T-cell responses. The level of HER-2/neu immunity was similar to viral and tetanus immunity. In addition, the peptide-specific T-cells were able to lyse tumor. The responses were long-lived and detectable for greater than 1 year after the final vaccination in selected patients. These results demonstrate that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER-2/neu-specific IFNγ-producing CD8+ T-cells.

### Stimulating a T helper cell response to HER-2/neu in vivo

Pre-existent immune responses to HER-2/neu are of low magnitude. Therefore, before an assessment as to the anti-tumor effect of HER-2/neu-specific immunity can be made, the level of immunity should be augmented. Stimulating an effective T helper response is a way to boost antigen-specific immunity as CD4+ T-cells generate the specific cytokine environment required to support an evolving immune response. Furthermore, either CTL or an antibody immunity may have an effect on HER-2/neu-overexpressing tumor growth. Targeting CD4+ T-cells in a vaccine strategy would result in the potential to augment either of these arms of the immune system.

Putative T helper subdominant peptide epitopes, derived from the HER-2/neu protein sequence, were predicted by computer modeling and screened for immune reactivity using PBMC from patients with breast and ovarian cancer (Disis & Cheever 1998). Vaccines were generated each composed of three different 15–18 amino acid long HER-2/neu peptides. Patients with advanced stage HER-2/neu-overexpressing breast, ovarian, and non-small-cell lung cancer were enrolled and 38 patients finished the planned course of 6 immunizations (Disis et al. 2002a). Patients received 500 µg of each peptide admixed in GM-CSF in an effort to mobilize LC in vivo as an adjuvant to peptide immunization (Disis et al. 1996a). Ninety-two percent of patients developed T-cell immunity to HER-2/neu peptides and over 60% to a HER-2/neu protein domain. Thus, immunization with peptides resulted in the generation of T-cells that could respond to protein processed by APC. Furthermore, at 1 year follow-up, immunity to the HER-2/neu protein persisted in 38% of patients. Immunity elicited by active immunization with CD4+ T helper epitopes was durable.

An additional finding of this study was that epitope spreading was observed in 84% of patients and significantly correlated with the generation of HER-2/neu protein-specific T-cell immunity ($P = 0.03$). Epitope, or determinant spreading, is a phenomenon first described in autoimmune disease (Lehmann et al. 1992) and has been associated with both MHC class I- and MHC class II-restricted responses (Vanderlugt & Miller 1996, el-Shami et al. 1999). Epitope spreading represents the generation of an immune response to a particular portion of an immunogenic protein and then the natural spread of that immunity to other areas of the protein or even to other antigens present in the environment. In this study, epitope spreading reflected the extension of a significant T-cell immune response to portions of the HER-2/neu protein that were not contained in the patient’s vaccine. How does epitope spreading develop? Theoretically, a broadening of the immune response may represent endogenous processing of antigen at sites of inflammation initiated by a...
specific T-cell response or ‘driver clone’ (Sercarz 2000). That is, the initial immune response can create a micro-environment at the site of the tumor that enhances endogenous immune effector cells present locally. These immune cells, e.g. APC and T-cells, may begin to respond more effectively to tumor antigen that is present in the body. Another recently reported vaccine trial immunizing breast and ovarian cancer patients with autologous DC pulsed with mucin-1 or HER-2/neu peptides resulted in epitope spreading (Brossart et al. 2000). In this trial, 10 patients were immunized and half the patients developed CD8+ T-cell precursors to their immunizing peptides. Moreover, some patients developed new immunity to other tumor antigens expressed in their cancers, such as CEA and MAGE-3.

Most clinical trials of cancer vaccines focus on the detection of a newly generated immune response or the magnitude of the antigen-specific immune response elicited after active immunization. However, the detection of epitope spreading indicating an immune microenvironment capable of producing an endogenous polyclonal immune response may be an endpoint that could potentially reflect an improved clinical outcome. Recent studies have evaluated vaccine strategies focused to maximize the role of the most efficient APC, the DC or skin LC, in eliciting effective immunity to self. One such strategy is to use cytokines involved in DC production and maturation as vaccine adjuvants. Flt3-ligand (FL) is a cytokine which, when administered systemically, can increase numbers of circulating DC greater than 40-fold (Maraskovsky et al. 2000). Human DC generated by the administration of FL have been shown to be functional and can stimulate T-cells in vitro (Maraskovsky et al. 2000). Furthermore, activation of DC in vivo by FL has been shown to be an effective way of circumventing tolerance during active immunization in animal models (Pulendran et al. 1998). Studies have been performed in the neu transgenic mouse, immunizing the animals to a self-tumor antigen, neu, using FL as a vaccine adjuvant to mobilize DC in vivo (Smorlesi et al. 1999). The timing of vaccine administration corresponded to the kinetics of in vivo DC mobilization in animals (Maraskovsky et al. 1996, Lynch 1998): early administration when few circulating DC are present, mid-point administration when DC precursors are increasing in the peripheral blood, and finally vaccination at the end of the FL cycle when DC are at peak concentrations. Thus, during a 10-day administration of FL a HER-2/neu ICD protein vaccine was administered at 3 time-points. Animals receiving the vaccine at mid-point in the FL cycle generated HER-2/neu ICD-specific immunity whereas mice immunized at the end of the FL cycle did not. In general, neu-specific immunity generated using FL resulted in T-cells that predominantly secreted IFNγ, a Type 1 associated cytokine, rather than interleukin (IL)-4, a Type 2 associated cytokine (Smorlesi et al. 1999).

On the basis of these data generated in rodent models, 10 patients with HER-2/neu-overexpressing cancers were enrolled to receive a HER-2/neu peptide-based vaccine targeting the ICD of the HER-2/neu protein (Disis et al. 2002b). The peptides in the vaccine were the same as those used in one of the arms of the trial described above (Disis et al. 2002a) admixed with GM-CSF alone as an adjuvant. All patients received FL 20 µg/kg per day s.c. for 14 days. Five patients received the HER-2/neu peptide-based vaccine alone intradermally at midpoint in one FL cycle and 5 patients received the vaccine admixed with 150 µg GM-CSF intradermally at midpoint in the FL cycle. T-cell proliferative responses to HER-2/neu peptides and ICD protein were not significantly boosted in either FL arm. However, including FL as a vaccine adjuvant was effective in boosting the precursor frequency of IFNγ-secreting HER-2/neu-specific T-cells. After the completion of all immunizations, all 4 patients in each group developed detectable IFNγ-producing T-cells specific for the ICD protein: FL alone arm, mean frequency 1:5000 (range 1:30 000–1:2000) and FL and GM-CSF arm, mean frequency 1:2500 (range 1:5700–1:1500). The small sample size of each group, however, did not allow a statistically significant comparison of immune responses between the FL alone and FL with GM-CSF arms.

Recent investigations have demonstrated that FL and GM-CSF may stimulate different subsets of DC in vivo and that the cytokine microenvironment elicited, either Type 1 or Type 2, is markedly influenced by the particular DC subset generated. Evaluating a murine model of cancer using tumors engineered to express either GM-CSF or FL, demonstrated that GM-CSF engineered cells were more potent in inducing an anti-tumor response (Mach et al. 2000). GM-CSF elicited a diverse cytokine environment consisting of both Th helper cell (Th) 1 and Th2 immune effectors. In contrast, immune responses generated with FL-expressing tumor cells were specifically restricted to a Th1 phenotypic response (Mach et al. 2000). Data from human clinical trials using FL as a vaccine adjuvant support the notion that FL is associated with the development of a strong Type 1 response. The detection of antigen-specific cytokine production without concomitant measurable clonal proliferation has been reported and is potentially a reflection of a strongly restricted Type 1 response (van der Veen et al. 1999). However, the increased number of APCs stimulated through the use of FL did function to present antigen and was verified by an additional finding. The addition of FL in the vaccine regimen was associated with the development of autoimmune phenomena in some patients. In general, the vaccine regimens including FL were well tolerated. One patient had grade 1 serologic abnormalities (anti-nuclear antibody (ANA), anti single strand antibody (anti-SSA), anti double strand DNA (anti-dsDNA)). The second patient, who had stage IV breast cancer, developed grade 2 toxicity with serologic abnormalities and self limiting Sicca syndrome characterized by dry eyes and dry mouth 3 months after the completion of the vaccine regimen. This patient did not develop any detectable immunity to HER-2/
neu peptides or protein after active immunization. None of the patients immunized on any reported HER-2/neu-specific vaccine trial developed any evidence of autoimmune phenomenon directed against tissues that express basal levels of HER-2/neu.

Active immunization results in increased precursor frequency and diversity of the T-cell repertoire

The generation of HER-2/neu antigen-specific T-cells from unprimed individuals is difficult. Isolation and expansion of T-cells from the peritoneal fluid of patients with HER-2/neu-overexpressing ovarian cancer require multiple in vitro stimulations (IVS) (Peoples et al. 1995, Fisk et al. 1996). In addition, deriving HER-2/neu peptide-specific T-cells from unprimed donors entails laborious and lengthy expansion techniques (Disis et al. 1994). By boosting HER-2/neu precursor frequency after peptide immunization, one can more readily expand and clone HER-2/neu-specific T-cells from the peripheral blood of patients with HER-2/neu-overexpressing breast cancers as compared with naive donors (Knutson & Disis 2001). The evaluation of a significant number of antigen-specific T-cells after active immunization (Peoples et al. 1995) will begin to allow us to dissect the tumor antigen-specific immune response for clues concerning the nature of the tumor-specific T-cells, affinity to MHC receptor, and potential for therapeutic efficacy.

A recent investigation demonstrated the marked phenotypic diversity of the vaccinated response (K L Knutson & M L Disis, unpublished observations). T-cell clones specific for HER-2/neu HLA-A2 peptide p369–377 were isolated from an ovarian cancer patient who had been vaccinated with HER-2/neu helper epitopes that contained HLA-A2-binding CTL epitopes within their sequences. Throughout the course of immunization, PBMC from this patient showed strong proliferative responses to the HER-2/neu helper epitope, p369–384. Following vaccination, T-cell clones specific for p369–377, the HLA-A2 binding peptide, were isolated by limiting dilution and then characterized. The responding T-cell repertoire generated was both phenotypically and functionally diverse. A total of 21 p369–377 T-cell clones were isolated from this patient. Sixteen of the clones were CD8+ and 5 of the clones were CD4+, despite being generated with an HLA-A2-binding peptide. The CD4 molecule is known to play a critical role in stabilizing the interaction of HLAA class II peptide with the T-cell receptor (TCR) and its presence promotes the expansion of low-affinity peptide-specific TCRs and ensures a diverse T-cell response. Although CD4+ T-cells are predominantly associated with responding to peptides associated with HLA class II, at lower frequencies CD4 plays a role in regulating HLA class I-restricted T-cells, particularly T-cells associated with cancers such as melanoma, colon, and pancreatic cancer (de Vries & Spits 1984, Somasundaram et al. 2000). Nineteen of 21 clones expressed the αβ TCR. The remaining 2 clones expressed the γδ TCR. Clones could lyse HLA-A2-transfected HER-2/neu-overexpressing tumor cells as well as peptide-loaded HLA matched cells. In addition to their lytic capabilities, these clones could be induced to produce IFNγ specifically in response to p369–377 peptide stimulation. The γδ TCR clones expressed CD8 and lysed HLA-A2 HER-2/neu-positive tumor cells, but not HLA-A2-negative HER-2/neu-overexpressing tumor cells. γδ T-cells are involved in a wide range of immune responses to infectious and non-infectious diseases, including malaria, mycobacterial infections, cancers, as well as autoimmune disorders such as multiple sclerosis (Boismenu & Havran 1998). Often, γδ T-cells clones are isolated from the tumor-infiltrating lymphocyte population of many tumors, including dysgerminoma (Zhao et al. 1995), seminoma (Zhao et al. 1995), renal carcinoma (Choudhary et al. 1995), lung (Yu et al. 1999), colorectal (Watanabe et al. 1995), and melanoma (Bachelez et al. 1992). The recruitment and role of these unique cells in mediating antitumor immunity is unknown. In autoimmune diseases, some pathological observations have been attributed to infiltrating γδ TCR T-cells. It is unknown if the autoreactive γδ TCR T-cells respond secondarily to damaged and stressed tissue (Hayday & Geng 1997) or if they initiate autoimmunity directly. One hypothesis is that, given their broad range of regulation by multiple mechanisms of antigen presentation and natural localization to epithelial tissue, γδ TCR T-cells are sentinels for the immune system and are capable of alerting the immune system to the presence of danger (e.g. infection, tumors, etc.). These results suggest that a tumor antigen-specific T-cell response can be markedly polyclonal at multiple levels, including T-cell subset and TCR. Perhaps functional immunity directed against specific self-antigens mimics the pathogenic pathways of autoimmune disease more closely than anticipated.

Defining the clinical role of cancer vaccines

The cumulative data from the limited number of completed phase I clinical trials using HER-2/neu peptide-based vaccines to immunize against the HER-2/neu protein indicate that patients can be vaccinated against this self-tumor antigen. Vaccination offers a potential therapeutic strategy to prevent the relapse of disease by establishing an effective memory response targeting HER-2/neu. In addition, active immunization can provide a polyclonal T-cell population specific for the tumor antigen that can be expanded and used in adoptive immunotherapy. Extrapolating from the experience with infectious disease vaccines, active immunization has the greatest chance of therapeutic efficacy if used in a minimal disease state, not against a rapidly growing drug-resistant tumor. Preclinical investigations have demonstrated that eradicating established tumors in cancer patients will
require the generation of high levels of tumor-specific immunity, levels which cannot be achieved by vaccination but rather by infusion of competent T-cells, i.e. adoptive T-cell therapy (Cheever & Chen 1997). Extrapolating from infectious disease models, T-cell precursor frequencies after influenza immunization may range from 1:25 000–1:5000. However, during an active infection, the antigen-specific T-cell precursor frequency may achieve levels of 1:50 circulating T-cells. Clearly, vaccination can increase the number of immune T-cells capable of recognizing and responding to antigen. Repeated vaccination further increases the number of immune effector cells, but eventually a plateau of responsiveness is reached and repeated immunizations do not appreciably change this value. Adoptive transfer of T-cells has resulted in the infused cells representing 1:2 of the host’s lymphocytes (Cheever & Chen 1997). Adoptive immunotherapy may allow levels of immunity to be achieved that may be able to treat bulky disease.

**HER-2/neu-specific T lymphocytes for adoptive T-cell therapy**

Patients with established, rapidly growing tumors can have an impaired cellular and humoral immune system. Therefore, it might be difficult to activate immunological defense mechanisms by vaccination. The rationale of adoptive T-cell therapy is based on the attempt to circumvent this tolerizing tumor microenvironment by taking out the anergic, potentially tumor-reactive T-cells from the cancer-bearing patient and subsequently activating these T-cells *ex vivo*. Following expansion of tumor-reactive T-cells *in vitro*, great numbers of T-cells can be adoptively transferred to the immunosuppressed patient. In this way, a high frequency level of *ex vivo* activated tumor-reactive T-cells can be achieved *in vivo*. This level of immunity cannot be achieved by active immunization.

The therapeutic efficacy of adoptively transferred T-cells was first documented in bone marrow recipients at risk for virus infections or virus-induced malignancies. Infusion of cytomegalovirus (CMV)-specific CTL clones generated from HLA-matched bone marrow donors resulted in the reconstitution of protective T-cell immunity against CMV (Riddell et al. 1992). In bone marrow recipients, similar results were obtained for restoration of immunity against Epstein-Barr virus (EBV) by adoptive transfer of EBV-specific T-cells generated from the donor (Rooney et al. 1998).

Although transfer of T-cells is a passive transfer of immunity, this immunotherapeutic strategy can potentially activate the endogenous immune system. The transferred T-cells can induce a cascade of cellular interactions leading to the initiation of an endogenous immune response. It has recently been shown that the dynamic interaction of CTL, T helper cells and antigen-presenting DC is required for the initiation of an immune response essential for antigen-specific tumor rejection. Dendritic cells take up antigens released from dead tumor cells and subsequently process and present antigenic peptides in the context of HLA class I and class II molecules to CD8+ and CD4+ T-cells respectively. In this scenario, antigen-specific CD4+ T-cells provide direct and indirect help to CD8+ effector T-cells (Ridge et al. 1998, Albert et al. 2001). During this three-cell interaction, CD8+ CTLs are not a passive partner, but are able to activate naive CD4+ T helper cells (Stuhler et al. 1999). Therefore, adoptively transferred CD4+ T-cells may help pre-existing CD8+ CTLs, and *vice versa*.

Attempts to treat HER-2/neu-overexpressing tumors by adoptive transfer of HER-2/neu reactive T-cells have been limited due to the difficulty of generating and expanding autologous CTL and T-helper cells directed against the HER-2/neu antigen. As discussed, *ex vivo* expansion of HER-2/neu-specific T-cells may be facilitated by increasing starting numbers of cultured cells by active immunization. Recently, investigators have developed a protocol using DC retrovirally transduced with the HER-2/neu gene for specific stimulation of autologous peripheral blood lymphocytes (Bernhard et al. 2000, Meyer zum Buschenfelde et al. 2000). HER-2/neu-transduced DC were capable of presenting multiple epitopes and subsequently induced HER-2/neu-specific cytotoxic and helper T-cells in individual donors (Meyer zum Buschenfelde et al. 2001). Both HER-2/neu reactive CD8+ CTL and CD4+ T-helper cells could be elicited and cloned from a patient with advanced HER-2/neu-overexpressing breast cancer. One of the advantages of using genetically modified DC as APC is the simultaneous stimulation of CTL and T-helper cells recognizing the same antigen in context with different HLA molecules. Clinical trials are poised to examine the therapeutic effect of the infusion of HER-2/neu-specific T-cell clones in order to define the immunological and clinical effect of certain T-cell populations following transfer. In the future, adoptive transfer of HER-2/neu-specific T-cell lines might be preferred to T-cell clones that recognize a single epitope (Knutson & Disis 2001).

Currently, one of the most widely used methods for generating CTL *in vitro* is the use of peptide-pulsed DC as APC. One advantage of using peptide-pulsed DC is the ability to isolate T-cells recognizing subdominant epitopes to which T-cells may not be elicited by stimulation with genetically modified DC. This culture method, however, is often not successful in generating antigen-specific T-cells, due to the low frequency of peptide-specific T-cells even after repetitive IVS. Moreover, this method often promotes the growth of peptide-specific T-cells with low affinity TCR that are unable to lyse tumor cells. The disadvantages of this method can be circumvented by sorting low-frequency antigen-specific T-cells with a high affinity TCR using HLA/peptide fluorescent tetramers (Altman et al. 1996). Using tetramer-guided sorting, high avidity melanoma-reactive CTLs have been isolated from heterogeneous populations that efficiently lyse tumor cells (Yee et al. 1999, Dutoit et al. 2001). However,
other groups have shown that structural avidity does not necessarily translate into high functional avidity (Bullock et al. 2001). Using tetramer-guided sorting, it is possible to isolate tumor-reactive HLA-A2-restricted CTLs against the HER-2/neu-derived peptide, p369–377, both from healthy donors and from patients with breast cancer (Schmidt et al. 2001).

HER-2/neu-specific T-cells can be expanded ex vivo after active immunization

The optimal conditions for culturing T-cells are not yet defined. Exogenous addition of the cytokines IL-7 and IL-15 to the T-cell culture might prevent activation-induced cell death that occurs upon antigen stimulation in the presence of IL-2 (Lynch & Miller 1994, Marks-Konczalik et al. 2000). In addition, IL-12 may prove to be a useful cytokine for T-cell expansion. Studies have evaluated the use of IL-12 for the polyclonal proliferation of both influenza-specific CTL and HER-2/neu-specific CD4+ T-cells (K L Knutson & M L Disis, unpublished observations). T-cells were expanded from the blood of patients immunized with a vaccine that contained a helper epitope, p776–790, derived from the ICD of HER-2/neu. This particular epitope has been shown to be broadly restricted in response (Sotiriadou et al. 2001). While immunity to p776–790 could be readily measured in short-term cultures, cell line generation by multiple IVS with peptide and IL-2 as the only added cytokine resulted in loss of activity. The inclusion of IL-12, along with IL-2, restored antigen-specific proliferation in a dose-dependent fashion. The resulting p776–790-specific T-cells responded readily to antigen by proliferating and producing type I cytokines (IFNγ and tumor necrosis factor α). The increased proliferative response of the cultures was due, in part, to an increase in the number of HER-2/neu-specific T-cells. IL-12 inclusion in the culture media also resulted in the decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for ex vivo recovery and maintenance of antigen-specific CD4 T lymphocytes that would otherwise be lost by using IL-2 alone in combination with antigen. The further development of defined culture conditions for growing T-cells with a defined phenotype is warranted.

The ability to grow and expand antigen-specific T-cells that are functionally active in vitro is a prerequisite, but not a guarantee, for the clinical success of adoptive T-cell transfer. In vitro assays for documenting the efficacy of tumor-reactive T-cells by measuring cytotoxicity or cytokine release, are not able to predict the in vivo activity of these T-cells following transfer. Multiple factors playing a role during the interaction of effector and tumor cells might prevent T-cells, which efficiently kill tumor cells in vitro, from eradicating tumor cells in vivo (Dudley et al. 2001). On the other hand, T-cells that display low cytolytic function in vitro might be good ‘killers’ in vivo (Lynch & Miller 1994). One novel method of improving HER-2/neu-specific CTL function in vivo after infusion of cells is the use of concurrent trastuzumab therapy with adoptive immunotherapy.

Synergistic activity of HER-2/neu-specific CTL cells and a HER-2/neu-specific monoclonal antibody, trastuzumab

Lytic activity of separated HER-2/neu-specific CTL has been low, in part, because HER-2/neu is a self-antigen and T-cells may display a low affinity to T-cell receptors due to induction of tolerance (Fisk et al. 1995, Linehan et al. 1995, Meyer zum Buschenfelde et al. 2000). On this basis, the lytic potential of HER-2/neu-specific CTL could, theoretically, be improved by further increasing the number of HLA class I-bound peptides on tumor cells with the help of trastuzumab, an inhibitory antibody against HER-2/neu. Upon binding of trastuzumab, the HER-2/neu receptor is internalized and degraded, subsequently inhibiting HER-2/neu-mediated signal transduction and tumor cell growth (Carter et al. 1992, Hurwitz et al. 1995). As antibody-induced degradation of HER-2/neu is likely to be accompanied with increased numbers of HER-2/neu peptides presented with HLA molecules, investigators questioned whether trastuzumab-treated tumor cells were more susceptible to CTL-mediated lysis. Indeed, HER-2/neu reactive CTL clones lyse class I-matched, HER-2/neu-overexpressing tumor cells more efficiently after treatment with trastuzumab (Meyer zum Buschenfelde et al. 2002). The potentially synergistic activity of HER-2/neu-specific antibody and CTL encourages the development of HER-2/neu targeted immunotherapy using a combination of inhibitory antibodies and infused CTL for the treatment of patients with HER-2/neu-overexpressing tumors. Planned studies will focus on the toxicity and efficacy of adoptively transferred HER-2/neu-specific CTL with and without trastuzumab in patients with HER-2/neu-overexpressing breast cancer.

Conclusion

Early results of clinical trials actively immunizing cancer patients against HER-2/neu demonstrate that immunity can be generated and that immune responses persist over a period of time. Current vaccine trials have focused solely on the use of epitope- or peptide-based vaccines, largely due to the observation that peptide vaccine strategies could circumvent neu-specific tolerance in rodent models. The next generation of vaccine approaches will include protein-based vaccines, HER-2/neu antigen preparations loaded onto DC, and nucleic acid based formulations. Studies in rodent models exploring these strategies at a pre-clinical level are promising. Expansion of HER-2/neu-specific T-cell ex vivo following active immunization or in vitro culture with HER-2/neu-expressing DC may be a therapeutic option for treating advanced stage HER-2/neu-overexpressing tumors.
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References

Boismenu R & Havran WL 1998 Gamma delta T-cells in host defense and epithelial cell biology. Clinical Immunology and Immunopathology 8 121–133.
Bullock TN, Mullins DW, Coella TA & Engelhard VH 2001 Manipulation of avidity to improve effectiveness of adoptively transferred CD8(a)+ T-cells for melanoma immunotherapy in human MHC class I-transgenic mice. Journal of Immunology 167 5824–5831.


CTL against autologous and allogeneic carcinomas and melanomas. 


