Breast cancer vaccines: maximizing cancer treatment by tapping into host immunity

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Abstract

Optimizing standard treatment modalities for breast cancer has improved the outlook for women afflicted with it, but the fact that 40% still ultimately die from the disease highlights the need for new therapies. Remarkable advances in molecular immunology and biotechnology have created a unique opportunity for developing active vaccination strategies that engage the patient’s own immune system in the fight against breast cancer. Early clinical trials have established the safety and bioactivity of some breast cancer vaccine approaches, with a hint of clinical response. They have also highlighted the importance of elucidating the pharmacodynamic interactions between established therapies for breast cancer, such as tamoxifen, aromatase inhibitors, chemotherapy, the HER-2/neu-specific monoclonal antibody trastuzumab (Herceptin), and breast cancer vaccines. Preclinical studies have simultaneously defined the importance of developing targeted approaches for circumventing established immune tolerance to breast cancer during the vaccination process. The first strategies targeting the negative influence of CD4^+CD25^+ T regulatory cells and the CTLA-4 signaling pathway are just entering clinical testing in combination with tumor vaccines. Developing the most potent approach for activating antitumor immunity while maintaining the efficacy of standard approaches to breast cancer management will ensure that active immunotherapy is successfully integrated into the standard of care.

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Introduction

Improvements in the use of traditional breast cancer therapies have decreased the morbidity and mortality of breast cancer treatment, and improved the overall survival of women with early stage disease (American Cancer Society 2002). The development of targeted drugs such as aromatase inhibitors (anastrozole (Arimidex), letrozole (Femara), and exemestane (Aromasin)), fulvestrant (Faslodex), and trastuzumab (Herceptin) has improved the quality of life for women with advanced disease. Current adjuvant trials are likely to demonstrate that these newer therapeutics will add an additional survival benefit for women with early breast cancer. Despite these remarkable advances, approximately 40% of women continue to fail current primary management strategies for early breast cancer, and ultimately succumb to their disease (American Cancer Society 2002). Furthermore, although women with metastatic disease can enjoy a good quality of life on therapy, metastatic breast cancer remains incurable. The failure of current management approaches is generally attributed to the outgrowth of breast tumor cells that are inherently resistant to standard treatments. Together, these observations underscore the need for unique approaches that can either overcome or circumvent intrinsic mechanisms of resistance to standard therapies. Manipulating the immune system to recognize and eradicate breast tumor cells is a highly attractive alternative approach to disease management.

Passive immunotherapeutic strategies include the adoptive transfer of antigen-specific T lymphocytes, and the infusion of monoclonal antibodies specific for a given tumor antigen. Trastuzumab (Herceptin), a monoclonal antibody specific for HER-2/neu, is a passive immunotherapeutic with efficacy against the biologically distinct subset of breast cancers that overexpress HER-2/neu (Emens & Davidson 2004). Alternatively, active immunization with tumor
vaccines can be employed to engage the patient’s own immune system to eradicate mammary tumor cells (Emens & Jaffee 2003a). Active immunization offers multiple theoretical advantages over all other therapies, including low toxicity and exquisite specificity. More importantly, the potential for a sustained antitumor effect due to immunologic memory would obviate the requirement for prolonged, repetitive cycles of therapy. However, the clinical efficacy of therapeutic vaccination against micrometastatic or advanced breast cancer is at the same time limited by active, pre-existing and redundant mechanisms of immune tolerance, the antigenic variability that arises from the genetic instability of breast tumor cells themselves, and the extent of established disease. In this review we describe the fundamental features of the basic antitumor immune response, highlight the altered immunologic milieu present in patients with breast and other cancers, and then the different vaccination strategies that have been tested in breast cancer to date. Finally, we propose strategies for integrating breast cancer vaccines with traditional and/or novel therapeutics in combined modality treatment regimens that have additive or synergistic activity compared with their individual components.

The antitumor immune response

The basic immune response

The antitumor immune response represents the integrated sum of interacting cell types that mediate innate, non-specific immunity, and adaptive, antigen-specific immunity. The innate arm of the immune system broadly surveys for signs of danger (invading micro-organisms for example), and collaborates with the adaptive (antigen-specific) immune response to effect antitumor immunity. Natural killer (NK) cells and specialized T cells recognize and lyse stressed host cells (Diefenbach & Raulet 2002). Additionally, immature dendritic cells stand ready to mature upon activation by exposure to danger signals (Walker & Abbas 2002). This activates the co-stimulatory pathways critical for effective T cell activation, enabling the development of finely tuned antigen-specific immune responses as described below.

While somatic cells can only present endogenous antigens in the context of major histocompatibility complex (MHC) Class I molecules, mature dendritic cells take up and process antigens through distinct pathways that result in antigen presentation mediated by both MHC Class I and II (Germain 1995). The proteosome pathway processes proteins into 8 to 12 amino acid fragments, ultimately loading them onto surface MHC Class I molecules for presentation to CD8+ T cells. They are also specialized to take up exogenous proteins, processing them through a lysosomal pathway to generate peptide fragments of 10 to 25 amino acids that are presented to CD4+ T cells in the context of MHC Class II. It is the ability of mature dendritic cells to activate both CD4+ and CD8+ T cells in the context of proper T cell co-stimulation that allows them to orchestrate a more potent immune response than any other antigen-presenting cell (APC) (Huang et al. 1996). Activated CD4+ T cells provide help to maximize the humoral (antibody) response mediated by B lymphocytes, and the magnitude and durability of the CD8+ cytotoxic T cell response. The immune response is further integrated by the engagement of antigen-specific immunoglobulin with specific receptors on neutrophils, macrophages, and NK cells, resulting in an alternate path to cell-mediated cytotoxicity (antibody or complement dependent). The integration of innate and adaptive immune responses by dendritic cells and immunoglobulin molecules argues that synergistically engaging innate and antigen-specific immune effectors is likely to result in the most effective tumor rejection response.

Co-stimulatory pathways constitute an immunologic rheostat

A variety of mechanisms exists for fine-tuning the immune response. A growing family of T cell co-stimulatory molecules provides a system for delivering positive and negative signals for T cell activation, thus representing an immunologic rheostat (Table 1) (Pardoll 2002, Croft 2003). The B7 family is a system of receptor-ligand pairs characterized by a pair that transmits an activation signal and a counter-regulatory pair that transmits a negative signal. The prototype pathway is B7 binding to CD28, thereby promoting T cell activation. Complementing this activity is the binding of B7 to cytotoxic Tymphocyte antigen-4 (CTLA-4) to attenuate T cell responses. Newer members include B7-H1/B7-DC binding to an unknown co-stimulatory ligand and the counter-regulatory ligand PD1, B7-related protein-1 (B7-RP-1) binding to inducible costimulator (ICOS) (positive signal), B7-H4 binding to B and T lymphocyte attenuator-4 (BTLA-4) (negative signal), and B7-H3 binding to unknown ligands to mediate both positive and negative signals. While ligands that promote T cell activation are generally found on APC, those that attenuate T cell responses are typically found on APC and in peripheral tissues (Khoury & Sayegh 2004). Finally,
cytokines such as interleukin (IL)-2, IL-12, and IL-18 determine T cell phenotype, and IL-7 and IL-15 promote the activation and expansion of cytotoxic T lymphocytes (CTL). The tumor necrosis factor receptor family represents a second group of regulatory molecules that deliver positive signals promoting effective T cell activation, expansion, and/or survival; these include OX40/OX40 ligand, 41BB/41BB ligand, CD40/CD40 ligand (CD154), CD27/CD27 ligand (CD70), and LIGHT/LIGHT receptors (Croft 2003). Signaling through these molecules can also enhance CTL-mediated tumor immunity by overcoming immune tolerance.

Superimposed on this intricate signaling network is the influence of novel subsets of regulatory cells derived from the dendritic, myeloid, and T lymphocyte lineages. Under normal conditions, immature dendritic cells enforce peripheral tolerance to tissue-specific antigens, maintaining immunologic homeostasis. However, antigen presentation by these dendritic cells can also result in antigen-specific T cells with skewed cytokine and chemokine receptor profiles, disrupting both T cell trafficking and effector function (Walker & Abbas 2002). Under conditions of immune activation, myeloid suppressor cells (MSC) come into play. MSCs represent a mixed population of immature and mature myeloid cells that express Gr-1 and CD11b, and inhibit the activation and expansion of CTL (Serafini et al. 2004). Finally, regulatory T cells (Treg) have recently emerged as a major influence on the immune response (O’Garra & Vieira 2004). Naturally occurring CD4+CD25+ Treg constitute 5–10% of CD4+ lymphocytes in healthy adult mice and humans. These cells also express CTLA-4 and the glucocorticoid-induced tumor necrosis factor receptor, secrete IL-10 and transforming growth factor-β (TGF-β), and are specifically characterized by expression of the forkhead/winged helix transcription factor (FoxP3). These cells prevent autoimmunity, but also dampen the antitumor immune response. A similarly active population of Treg cells producing IL-10 and TGF-β that does not express FoxP3 has also been described.

### Immune dysregulation in breast cancer

There is substantial evidence for immune defects in patients with breast cancer. They have lower absolute numbers of peripheral blood lymphocytes (Caras et al. 2004), but increased numbers of functionally suppressive CD4+CD25+ Treg in the peripheral blood and tumor microenvironment (Liyange et al. 2002, Wolf et al. 2003). Dendritic cells obtained from peripheral blood and lymph nodes of patients with operable breast cancer are dysfunctional, with decreased levels of MHC Class II and CD86 (B7) expression, and IL-12 secretion. Cyclo-oxygenase-2-dependent and prostaglandin E2 secretion in the breast tumor microenvironment has been proposed as one mechanism for this diminished T cell and dendritic cell function (Pockaj et al. 2004). Complicating these issues of breast cancer biology is the inherent and dynamic phenotypic instability of breast tumor cells themselves. Since tumors arise endogenously and progress, a continual dialogue between the tumor phenotype and the immune response ensues, with each influencing the other to evolve (Dunn et al. 2004). Thus, tumors can downregulate the expression of tumor antigens targeted by a natural immune response, an antigen-specific vaccine, or a therapeutic antibody, resulting in antigen loss variants resistant to the therapeutic intervention (Davis et al. 1999, Riker et al. 1999, Knutson et al. 2004). This dynamic can be overcome

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Signal</th>
<th>Receiving cell</th>
<th>Reagents available for clinical use</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40/CD40 ligand</td>
<td>+</td>
<td>APC/T cell</td>
<td>Yes: anti-CD40 ligand antibodies for autoimmune disease</td>
</tr>
<tr>
<td>41BB/41BB ligand</td>
<td>+</td>
<td>APC/T cell</td>
<td>No</td>
</tr>
<tr>
<td>OX40/OX40 ligand</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>CD70/CD27</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>LIGHT/LIGHT receptor</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7/CD28</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7-H1;B7-DC/?</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7-RP-1/ICOS</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7-H3/?</td>
<td>+</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7/CTLA-4</td>
<td>–</td>
<td>T cell</td>
<td>Yes: MAbs CP-675,206 and MDX-010 for cancer immunotherapy</td>
</tr>
<tr>
<td>B7-H1;B7-DC/PD-1</td>
<td>–</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7-H3/?</td>
<td>–</td>
<td>T cell</td>
<td>No</td>
</tr>
<tr>
<td>B7-H4/BTLA-4</td>
<td>–</td>
<td>T cell</td>
<td>No</td>
</tr>
</tbody>
</table>

MAbs, monoclonal antibodies.
by choosing a target antigen that is requisite for the transformed phenotype, or by developing cancer vaccines that simultaneously target multiple tumor antigens. Adding further complexity to the host–tumor interaction, tumors can also down-modulate components of the antigen-processing pathways, including MHC Class I and Class II molecules, various proteasome subunits, and the transporter associated with antigen processing (TAP) (Marincola et al. 2000). The relevance of these altered antigen-processing phenotypes has been suggested by their correlation with poor clinical outcome (Kageshita et al. 1999).

Perhaps most importantly, it has become clear that it is critical to develop effective strategies for breaking or circumventing existing immune tolerance in the context of vaccination against cancer. In contrast to active immunization for infectious diseases, which elicits immunity to foreign antigens, vaccination for cancer treatment and prevention most often targets antigens that are perceived by the immune system as self. It is this fundamental difference that underlies the differential between observed T cell precursor frequencies in the setting of acute infectious diseases (>10%) as compared with tumors (<1%). Importantly, multiple regulatory mechanisms have evolved to minimize the immune response to antigens perceived as self (Walker & Abbas 2002). T cells with the highest affinity for self antigens are typically deleted in the thymus or peripherally, often leaving in place an alternative antigen-specific T cell repertoire with relatively low affinity for their target. This, combined with the fact that tumor antigens are most often detected in the absence of the co-stimulatory signals essential for immune activation, renders potentially active T cells unresponsive or anergic. Additionally, antigen-specific functional T cells can be rendered ignorant, where they simply fail to perceive cognate antigen either due to extremely low levels of expression or due to partitioning the antigen in an immunologically privileged site. Finally, as previously described, novel subsets of myeloid cells, dendritic cells, and regulatory T cells are also effective suppressors of the immune response. Understanding the host–tumor dynamic and the role of fundamental immunoregulatory mechanisms in shaping the available tumor-specific T cell repertoire should facilitate the development of unique combinatorial vaccination strategies that can maximize antitumor immunity.

Breast cancer vaccine approaches

Several types of breast cancer vaccines that engage distinct aspects of the antitumor immune response are at different stages of preclinical and clinical evaluation. Those that have been tested in early clinical trials to date are summarized in Table 2 (Emens & Jaffee 2003b). Additional vaccine platforms are under development, including plasmid DNA-based vaccines, vaccines comprised of recombinant viral vectors or recombinant bacteria incorporating tumor antigens, and heat-shock protein-based vaccines. Overall, these approaches can generally be classified into those that deliver a limited number of well-defined tumor antigens, and those that deliver a panel of antigens, some of which are defined and some of which are not. In general, current vaccination strategies have been developed to directly manipulate B cells, T lymphocytes, or professional APCs (usually dendritic cells). One strategy for directly activating humoral immunity is vaccinating with tumor-specific carbohydrate antigens delivered either by whole tumor cells or as conjugates with keyhole limpet hemocyanin (KLH). There are several approaches for directly activating tumor-specific T cells. One is immunization with whole tumor cells genetically modified to express co-stimulatory molecules or immune-activating cytokines. Another is by modifying APCs to directly express relevant tumor antigens. A third approach is to activate T cells indirectly by the sustained local delivery of cytokines, which recruits professional antigen-presenting cells to the site of antigen deposition (the vaccination site) in vivo. The early empiric analysis of a panel of immune-activating cytokines revealed granulocyte-macrophage colony-stimulating factor (GM-CSF) to be the most potent in this regard (Dranoff et al. 1993). Based on this observation, the use of GM-CSF-secreting tumor cells as cancer vaccines has been reported in Phase I clinical trials in melanoma (Soiffer et al. 1998, 2003), renal cell carcinoma (Simons et al. 1997), prostate cancer (Simons et al. 1999, 2001), pancreatic cancer (Jaffee et al. 2001), and non-small cell lung cancer (Salgia et al. 2003, Neumanits et al. 2004). We are currently conducting the first clinical trial of vaccination with GM-CSF-secreting breast tumor cells in patients with metastatic breast cancer (Emens et al. 2004). In the aggregate, these trials have demonstrated the safety and bioactivity of this vaccination strategy, with a suggestion of potential clinical benefit.

Clearly, the development of effective immunotherapies for breast cancer management will be greatly facilitated by the identification of immunologically relevant breast tumor antigens. Several breast tumor antigens have already been identified based on the biology of breast cancer, the identification of antigens to which patients with breast cancer naturally develop
antibody or T cell responses, or the identification of important tumor antigens expressed by other tumor histologies that are also expressed in breast tumors. These include Mucin-1 (MUC-1), HER-2/neu, carcinoembryonic antigen (CEA), p53, sialyl-Tn (STn), the melanoma-associated (-testis) antigens MAGE, BAGE, GAGE, and XAGE, and the putative universal tumor antigens survivin and telomerase (hTERT) (Emens & Jaffee 2003). Of these antigens, MUC-1 and HER-2/neu have been most extensively tested as vaccine targets. Notably, breast cancer patients can develop detectable, albeit low, levels of humoral and cellular immunity specific for MUC-1 and HER-2/neu (Disis et al. 1994, 1997, Miles & Papazisi 2003), suggesting that it may be possible to augment pre-existent MUC-1- or HER-2/neu-specific immune responses. Finally, the effectiveness of Herceptin, a humanized monoclonal antibody specific for HER-2/neu, against HER-2/neu-overexpressing metastatic breast cancers, defines it as the first validated immunologic target for breast cancer therapy (Cobleigh et al. 1999).

**Vaccines targeting HER-2/neu and MUC-1**

Given observations that breast cancer patients naturally develop low levels of MUC-1- and HER-2/neu-specific immunity, it is not surprising that

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**Table 2** Summary of Phase I and II breast cancer vaccine trials

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Adjuvant</th>
<th>Antigen-specific immune response</th>
<th>Clinical benefit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC-1 peptide-KLH</td>
<td>DETOX-B, CY</td>
<td>MUC-1-specific IgG, T cells</td>
<td>NR</td>
<td>Reddish et al. 1998</td>
</tr>
<tr>
<td>MUC-1 peptide-KLH</td>
<td>QS-21</td>
<td>IgM, IgG, ADCC KLH-specific T cells</td>
<td>No</td>
<td>Adluri et al. 1999, Gilewski et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No MUC-1-specific T cells</td>
<td></td>
<td>Snijdewint et al. 2001, Muselli et al. 2002</td>
</tr>
<tr>
<td>α-idiotype 11D10 (HMFG)</td>
<td>HDC/ASCT</td>
<td>Idiotype-specific antibody and T cells</td>
<td>No</td>
<td>Reece et al. 2003</td>
</tr>
<tr>
<td>α-idiotype 1E10 (NeuGcGM3-VSSP)</td>
<td>None</td>
<td>Idiotype/NeuGcGM3-specific antibody</td>
<td>NR</td>
<td>Diaz et al. 2003</td>
</tr>
<tr>
<td>GLOBO-H-KLH</td>
<td>QS-21</td>
<td>IgM, CDC, ADCC No IgG</td>
<td>56% 2-year disease-free progression</td>
<td>Gilewski et al. 2001</td>
</tr>
<tr>
<td>HER-2 peptide GM-CSF (Flt-3)</td>
<td>IgM, T cell, DTH</td>
<td></td>
<td>Prolonged TTP (trend $P = 0.06$)</td>
<td>Murray et al. 2002</td>
</tr>
<tr>
<td>HER-2 peptide GM-CSF</td>
<td>T cell, DTH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER-2 ICD protein GM-CSF</td>
<td>IgG, T cell</td>
<td>NR</td>
<td></td>
<td>Disis et al. 2004</td>
</tr>
<tr>
<td>HER-2-dendritic cell</td>
<td>None</td>
<td>T cell</td>
<td>20% PR ($n = 10$)</td>
<td>Dees et al. 2004</td>
</tr>
<tr>
<td>p53-dendritic cell</td>
<td>None</td>
<td>T cell</td>
<td>33% SD</td>
<td>Svane et al. 2004</td>
</tr>
<tr>
<td>NDV-autologous tumor</td>
<td>None</td>
<td>NR</td>
<td>Trend toward improved survival</td>
<td>Ahlert et al. 1997</td>
</tr>
<tr>
<td>Allogeneic tumor BCG</td>
<td>NR</td>
<td>31% 10-year DFS</td>
<td></td>
<td>Wiseman et al. 1995</td>
</tr>
<tr>
<td>CD80:MDA-MBA231 GM-CSF</td>
<td>Inconsistent</td>
<td>No</td>
<td></td>
<td>Dols et al. 2003a,b</td>
</tr>
<tr>
<td>DC-tumor fusion</td>
<td>T cell</td>
<td>43% SD/PR ($n = 23$)</td>
<td></td>
<td>Avigan et al. 2004</td>
</tr>
</tbody>
</table>

MUC-1 = mucin-1; KLH = keyhole limpet hemocyanin; ADCC = antibody-dependent cellular cytotoxicity; HMFG = human milk fat globule; HDC/ASCT = high dose chemotherapy with autologous stem cell transplant; CDC = complement-mediated cytotoxicity; CY = cyclophosphamide; GM-CSF = granulocyte-macrophage colony-stimulating factor; Flt-3 = flt ligand-3; DTH = delayed type hypersensitivity; ICD = intracellular domain; BCG = bacillus Calmette Guérin; NR = not reported; SD = stable disease; PR = partial response; DFS = disease-free survival; TTP = time to progression.

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HER-2/neu and MUC-1 have been most extensively tested as vaccine targets. HER-2/neu has been targeted with both dendritic cell (Morse et al. 2003)- and peptide-based vaccines, but far more data have been generated with the peptide platform. Disis and colleagues have evaluated these vaccines clinically in patients with Stage III or Stage IV HER-2/neu-overexpressing breast, ovarian, or non-small cell lung cancer (Disis et al. 1999, 2000, 2002a,b, Knutson et al. 2001, 2002). Using computer modeling and empiric testing, they identified candidate peptide epitopes capable of eliciting either MHC Class II-restricted (CD4+ T cell) responses, MHC Class I-restricted (CD8+ T cell) responses, or both. The candidate epitopes were combined in alternative formulations with either GM-CSF or Flt-3 as vaccine adjuvants to promote the recruitment and activation of dendritic cells, and thus enhanced antigen presentation and immune priming. The largest clinical trial tested the ability of a multipeptide vaccine formulated with GM-CSF to elicit CD4+ T cell responses (Disis et al. 2002a). Thirty-one of thirty-eight research subjects who completed 6 monthly vaccinations had Stage III or IV breast cancer. Ninety-two percent of the patients completing six vaccinations developed HER-2/neu immunity to at least one peptide component of the vaccine as measured by peptide-specific T cell proliferation in vitro. Three observations support this in vitro assay as a correlate of robust HER-2/neu-specific immune responses. First, it is associated with epitope spreading to relevant HER-2/neu peptide fragments not delivered by the vaccine itself. Secondly, it is associated with the development of significant HER-2/neu peptide-specific delayed type hypersensitivity (DTH; >1.0 cm), reflecting the ability of the vaccine-induced T cells to traffic to the site of antigen deposition in vivo (Disis et al. 2000). Finally, HER-2/neu-specific immunity persisted for at least 1 year in 38% of responders, illustrating the durability of vaccine-activated immunity in some patients. Murray et al. (2002) independently evaluated the human leukocyte antigen (HLA)-A2-restricted HER-2-derived peptide p369-377 given with GM-CSF to 13 patients with metastatic breast or ovarian cancer. They also observed the development of new peptide-specific DTH in seven patients, four of whom developed antigen-specific CTLs capable of lysing HER-2/neu-expressing tumors.

These findings were extended in a recently reported clinical trial testing escalating doses of a protein-based vaccine composed of the HER-2/neu intracellular domain in patients with Stage II, III or IV breast cancer and no evidence of disease (Disis et al. 2004). Over 80% of vaccinated individuals developed HER-2/neu-specific T cell and humoral immunity consistent with the priming of both CD4+ and CD8+ T cell responses. The magnitude of response was unaffected by vaccine dose, although the time to immune response was shorter with the higher dose of protein. Thus, HER-2/neu-directed vaccination is safe, and associated with the induction of antigen-specific immunity in early clinical trials.

Multiple clinical trials have also tested the safety and bioactivity of vaccines that target MUC-1-derived peptide or carbohydrate epitopes. One group of trials tested a MUC-1 tandem repeat peptide-KLH conjugate given with the immunologic adjuvant QS-21 (a saponin) to patients with stable metastatic breast cancer (Adluri et al. 1999, Gilewski et al. 2000, Snijders et al. 2001, Muselli et al. 2002). This vaccination strategy induced antigen-specific IgM and IgG antibody titers associated with NK-directed antibody-dependent cellular cytotoxicity (ADCC) as measured in vitro. Although concomitant KLH-specific T cell immunity was also induced, no evidence of MUC-1-specific T cell immunity was found. A second group of trials evaluated the MUC-1 STn carbohydrate epitope conjugated to KLH (THERATOPE, Biomira Inc., Edmonton, Alberta, Canada) given with the immunologic adjuvant DETOX-B (Enhanzyn, Corixa Corp., Seattle, WA, USA); some vaccinees also received cyclophosphamide intravenously 3 days prior to vaccination (Miles et al. 1996, Ibrahim & Murray 2003). Phase I and II studies revealed the development of new STn-specific IgG and IgM, with higher antibody titers and longer median survival associated with cyclophosphamide pretreatment. Based on these data, a multicenter, randomized, double-blind Phase III study of 1028 randomized THERATOPE-treated patients with stable metastatic breast cancer to cyclophosphamide and THERATOPE (STn-KLH) or cyclophosphamide and control (KLH alone) was carried out (Fig. 1) (Ibrahim et al. 2004, Mayordomo et al. 2004). Thirty-four percent of participants were on concurrent hormone therapy. Although no differences in time to disease progression (TTP) or overall survival (OS) emerged in an intent to treat analysis, an exploratory analysis revealed a trend toward improved TTP and OS in those participants on hormone therapy. Median OS was greater in the subgroup of patients receiving hormone therapy who developed higher than median IgG titers specific for naturally clustered STn antigens (asialo-ovine submaxillary mucin (OSM)), with median OS of 41.1 months versus 25.4 months respectively (log-rank P=0.01) (Table 3). The study remains in follow-up.
What have we learned from early clinical trials of breast cancer vaccines?

The first clinical trials of breast cancer vaccines have defined a set of principles for future vaccine development. First, the HER-2/neu peptide vaccine trials illustrate that it is possible to prime both CD4+ and CD8+ T cell responses. Secondly, the MUC-1 trials demonstrate that antibody responses alone do not appear to correlate with clinical responses, at least not in the case of the MUC-1 antigen. Of equal importance, the THERATOPE vaccine trials argue that vaccines as a single intervention are unlikely to have a significant impact on disease outcome. Thus, innovative combinatorial approaches for enhancing antigen-specific immune responses and incorporating active

Table 3 Humoral immune responses and median overall survival in the Phase III THERATOPE vaccine trial. Data are given as median OS in months, with the number of patients in each group given in parentheses

<table>
<thead>
<tr>
<th>Intervention</th>
<th>THERATOPE ITT</th>
<th>THERATOPE with HT</th>
<th>THERATOPE without HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-OSM IgG titer</td>
<td>High: 31.2 (242)</td>
<td>High: 41.4 (98)</td>
<td>High: 23.7 (144)</td>
</tr>
<tr>
<td></td>
<td>Low: 24.0 (125)</td>
<td>Low: 25.4 (45)</td>
<td>Low: 23.0 (80)</td>
</tr>
<tr>
<td></td>
<td>( P = 0.08 )</td>
<td>( P = 0.01 )</td>
<td>( P = 0.92 )</td>
</tr>
<tr>
<td>Anti-STn IgG titer</td>
<td>High: 30.3 (210)</td>
<td>High: 41.1 (82)</td>
<td>High: 23.7 (128)</td>
</tr>
<tr>
<td></td>
<td>Low: 26.4 (157)</td>
<td>Low: 35.0 (61)</td>
<td>Low: 23.0 (96)</td>
</tr>
<tr>
<td></td>
<td>( P = 0.57 )</td>
<td>( P = 0.36 )</td>
<td>( P = 0.97 )</td>
</tr>
</tbody>
</table>

Secondary endpoints of the Phase III THERATOPE vaccine trial were humoral (IgG) responses to KLH (data not shown), the STn antigen (the synthetic monomeric form delivered by the vaccine), and OSM antigen (naturally occurring clustered STn antigens). IgG was measured by ELISA at 12 weeks, and median survival assessed as a function of IgG titers. Median overall survival was associated with the development of significant anti-OSM titers only. ITT = intent to treat analysis; HT = hormone therapy. Adapted from Ibrahim et al. (2004).
immunotherapy with standard breast cancer management (concurrent hormone therapy) will likely be required. Additionally, the administration of cancer vaccines is generally associated with minimal toxicity that is largely limited to local injection site reactions. The design of future Phase I and II vaccine trials should thus give equal weight to the assessment of vaccine safety and bioactivity. Formulating vaccines that contain defined antigens representing true tumor rejection targets or surrogate markers of effective antitumor immune responses for monitoring vaccine-induced immunity is essential. Together, these principles strongly argue for testing tumor vaccines in patients with minimal to no evidence of disease. One such approach is to incorporate them as one component of adjuvant therapy for patients who are at high risk for relapse. An alternative strategy is to test breast cancer vaccines as part of combined modality treatment regimens in patients with established metastatic disease that has been optimally debulked with standard therapies. Regardless, the effective design of trials for either patient population will require the consideration of the pharmacodynamic interactions between standard treatment modalities and vaccine therapy.

Combinatorial vaccination regimens
Integrating breast cancer vaccines into established treatment strategies will hasten their development for both treatment and prevention. Specifically, breast cancer vaccines can be combined with systemic breast cancer therapies to capitalize on both their cytoreductive and immunomodulatory activity. As illustrated with the use of low-dose cyclophosphamide (300 mg/m²) with the THERATOPE vaccine, chemotherapy in particular can be used in novel ways to modulate the immune response. Moreover, therapeutic monoclonal antibodies such as trastuzumab (Herceptin) also appear to augment the innate and adaptive antitumor immune response (Klapper et al. 2000, Castilleja et al. 2001, zum Buschenfelde et al. 2002). Here we review the clinical and preclinical data supporting the early testing of breast cancer vaccines, not as single therapeutic agents, but in sequence or in combination with traditional therapeutic modalities in an additive or synergistic fashion.

Cancer vaccines and chemotherapy
MUC-1-based vaccines have been tested in the setting of high-dose chemotherapy (HDC) with autologous stem cell transplant (ASCT) in two small trials. THERATOPE was administered to 70 patients with either breast (n=53) or ovarian (n=17) cancer in the setting of HDC with ASCT (Holmberg et al. 2003). Study subjects developed STn-specific IgG and T cells, but the study was not adequately powered to determine the relationship between immunization and clinical benefit. A distinct type of MUC-1-targeted vaccine employs the anti-idiotype monoclonal antibody 11D10 (TriAbR) to induce MUC-1-specific immune responses (Reece et al. 2003). This antibody mimics an MUC-1-like epitope present in the human milk fat globule (HMFG) protein found in over 90% of breast cancers. Since the idiotypic determinant contained within the antigen-binding site of the antibody reflects the true antigenic epitope, vaccination with 11D10 should result in the production of anti-anti-idiotype antibodies that recognize the HMFG tumor antigen. Fifty-four patients with chemosensitive metastatic breast cancer were immunized with 11D10 in the context of HDC with ASCT. Vaccine was given 1 week after the last cycle of conventional chemotherapy, then weekly for three doses prior to priming for peripheral blood stem cell collection. After HDC and ASCT, vaccination was resumed and continued monthly for up to 2 years. Idiotype-specific humoral and T cell proliferative responses were observed in the majority of patients, peaking at 3.5 months and 4.8 months after ASCT respectively; there was no clinical benefit conferred by vaccination. Although the relevance of these studies to the current management of breast cancer is low, the results suggest that it may be possible to induce antigen-specific immunity in close proximity to significant doses of chemotherapy.

In contrast, other data have clearly demonstrated a detrimental effect of standard dose chemotherapy on vaccine-activated, antigen-specific immunity. Both a greater number of prior chemotherapy regimens and close proximity to a prior chemotherapy treatment limited the induction of CEA-specific T cell precursors in patients with advanced colorectal carcinoma immunized with a canary pox vaccine (ALVAC-CEA) (von Mehren et al. 2001). Moreover, a second study testing an allogeneic GM-CSF-secreting pancreatic tumor vaccine in patients with Stage II or III pancreatic cancer after pancreatectoduodenectomy also demonstrated an adverse effect of aggressive adjuvant therapy on vaccine-activated immunity (Jaffee et al. 2001). Study subjects were immunized immediately after surgery, then monthly for 3 months after completing 6 months of adjuvant chemoradiation. The serial enzyme-linked immunospot (ELISPOT) analysis of postvaccination antigen-specific CD8⁺ T cell responses
in three responders demonstrated that T cell precursor frequencies established by the first vaccination decayed during chemoradiation, and were not restored by subsequent vaccination until the three booster vaccinations were completed (Thomas et al. 2004).

The difference between the interaction of vaccines with high-dose as compared with standard-dose chemotherapy may be related to the association of lymphopenia-induced homeostatic T cell proliferation with high-dose, but not standard-dose, chemotherapy. Lymphopenia-induced homeostatic T cell proliferation is a recently described mechanism for restoring the memory T cell compartment (Cho et al. 2000, Goldrath et al. 2000). Preclinical data suggest that manipulating the T cell repertoire with cancer vaccines during immune reconstitution after lymphoablative treatment might skew the immune system toward a desired tumor specificity (Mackall et al. 1996). For example, melanoma-specific T cells could be selectively expanded by immunization with a GM-CSF-secreting melanoma vaccine in lymphopenic (Rag-1-deficient) mice to induce the rejection of pre-established tumors (Hu et al. 2002). These results are recapitulated with the administration of GM-CSF-secreting tumor vaccines during early engraftment after syngeneic or allogeneic T cell-depleted bone marrow transplantation in murine models (Borrello et al. 2000, Teshima et al. 2001). While the phenomenon of homeostatic proliferation has not yet been rigorously demonstrated in patients, two recent trials testing the adoptive transfer of tumor-specific lymphocytes in the setting of non-myeloablative chemotherapy (or not) for melanoma suggest that it could be clinically relevant (Dudley et al. 2002, Yee et al. 2002). Yee et al. (2002) adoptively transferred melanoma-specific CD8+ T cell clones combined with low-dose IL-2 to ten patients with metastatic melanoma; the patients did not receive conditioning therapy prior to adoptive transfer. While transferred T cells localized to tumor sites, only minor, mixed, or stable responses were observed in eight patients. Dudley et al. (2002) treated 13 patients with metastatic melanoma with cyclophosphamide and fludarabine, then adoptively transferred a mixture of highly selected CD4+ CD8+ T cells derived from tumor-infiltrating lymphocytes (TIL) with high dose IL-2. Six individuals had more than 50% reduction at all tumor sites. Two of these had persistent, stable engraftment of transferred TIL, which accounted for over 70% of circulating CD8+ effector memory T cells. These trials suggest the potential influence of the lymphopenic host environment on the efficacy of immunotherapy, and an important contribution of CD4+ T cells to the ensuing immune response. Since many traditional cancer therapies result in lymphopenia, carefully delineating their influence on the kinetics, persistence, and functional quality of antigen-specific immune reconstitution will be required for the effective application of breast cancer vaccines to the lymphopenic setting.

Chemotherapy as a vaccine adjuvant

In addition to the established cytotoxic and immuno-suppressive activities of most chemotherapeutic agents, some also either augment or abrogate antigen-specific immune responses depending on the drug dose and timing of administration in relation to antigen challenge (Emens et al. 2001). The ability of low-dose cyclophosphamide given prior to the THERATOPE vaccine to augment STn-specific immune responses in early clinical trials led to the wide testing of this sequence in the Phase III clinical trial. In addition to cyclophosphamide, a number of other chemotherapeutic agents, including paclitaxel, doxorubicin, methotrexate, 5-fluorouracil, cytarabine, cisplatinum, doxorubicin, and cyclophosphamide (Nigam et al. 1998). Although the majority of drugs reduced vaccine activity in this model system, doxorubicin clearly augmented antigen-specific immune responses. Low-dose doxorubicin (2–6 mg/kg) given at the time of or subsequent to vaccination augmented CT-26-specific CD8+ T cell immunity, whereas doxorubicin pretreatment abrogated vaccine activity. Doxorubicin plus vaccination cured 40% of tumor-bearing mice, whereas cyclophosphamide (50–250 mg/kg) plus vaccination or vaccination alone cured 30–35% of tumor-bearing mice. In contrast to doxorubicin, cyclophosphamide inhibited vaccine activity when given at the time of or subsequent to vaccination.

We extended this approach to a model of profound antigen-specific immune tolerance relevant to breast cancer, the HER-2/neu transgenic mouse. As the result of mouse mammary tumor virus (MMTV)-driven expression of the rat proto-oncogene neu, these mice spontaneously develop HER-2/neu-overexpressing breast cancers (Guy et al. 1992). At baseline, they demonstrate very low levels of HER-2/neu-specific antibody titers and T cell immunity similar to that observed in patients with HER-2/neu-overexpressing breast cancer (Reilly et al. 2000). Importantly, whereas tumor-bearing parental FVB/N mice vigorously reject
HER-2/neu-expressing tumors after HER-2/neu-targeted, GM-CSF-secreting whole cell vaccination, the tumor outgrowth rates of vaccinated tumor-bearing neu transgenic mice are not impacted at all by immunization alone (Reilly et al. 2000). This striking difference in vaccine efficacy illustrates the impact of HER-2/neu-specific immune tolerance in this system. Notably, giving a HER-2/neu-specific vaccine in timed sequential fashion with cyclophosphamide, paclitaxel, or doxorubicin partially overcomes immune tolerance, delaying tumor outgrowth compared with those neu transgenic mice treated with vaccine alone (Machiels et al. 2001). Specifically, cyclophosphamide (100 mg/kg) or paclitaxel (20 mg/kg) given 1 day prior to vaccination significantly delayed tumor outgrowth compared with either drug or chemotherapy alone. Conversely, sequencing the vaccine with doxorubicin (5 mg/kg) 1 week after vaccination also resulted in an enhanced antitumor response. Importantly, the positive interaction between drug and vaccine diminished with nadiring T cell counts, arguing against homeostatic proliferation as the mechanism of augmented vaccine activity in the neu mouse model. Moreover, vaccine activity was abrogated by doxorubicin pre-treatment or the administration of cyclophosphamide or paclitaxel postvaccination. The immunomodulatory activity of the drugs was confirmed by their ability to interact with vaccination, effecting the rejection of a subsequent tumor challenge and increasing the numbers of HER-2/neu-specific CD4\(^+\) T helper type I cells as measured by ELISPOT. Timed sequential therapy with cyclophosphamide on day −1, vaccination on day 0, and doxorubicin on day +7 demonstrated the greatest immunologic and therapeutic potency, curing up to 30% of mice of pre-established tumors. Similar results have been reported when doxorubicin or paclitaxel treatment was given prior to HER-2/neu-targeted virus replicon-based vaccines (Eralp et al. 2004). Based on these data, we are currently conducting a Phase I clinical trial testing an allogeneic GM-CSF-secreting cellular breast cancer vaccine given in a timed sequence with low, immunomodulatory doses of cyclophosphamide and doxorubicin to patients with metastatic breast cancer (Emens et al. 2004).

These drugs modulate the induction of antigen-specific immunity in distinct ways. Cyclophosphamide can overcome natural and acquired immune tolerance if given before an antigen exposure, but promotes the induction of immune tolerance if given concomitantly with antigen (Emens et al. 2001). Cyclophosphamide is associated with the production of type I interferons, CD4\(^+\) T cell immunity of the T helper type I phenotype, and increased numbers of CD44\(^ HI\) memory T cells (Schiavoni et al. 2000). Cyclophosphamide has also been demonstrated to block the activity of CD4\(^+\)CD25\(^+\) regulatory T cells (Ghirlingelli et al. 2004); our own unpublished data are consistent with this. Like cyclophosphamide, paclitaxel promotes the priming of antigen-specific CD4\(^+\) T cell with the T helper type I phenotype (Machiels et al. 2001). It mimics the effect of lipopolysaccharide, enhancing macrophage activity through the induction of nitric oxide activity and other pro-inflammatory mediators (Chan & Yang 2000). It may also enhance antigen presentation by specifically binding to toll-like receptors on dendritic cells (Kawasaki et al. 2000, Byrd-Leifer et al. 2001, Wang et al. 2002), and by promoting tumor cell apoptosis and the development of tumor-infiltrating lymphocytes (Hal dar et al. 1997, Demaria et al. 2001). Both taxanes also augment T cell blastogenesis as well as NK- and lymphokine-activated killer cell function (Tong et al. 2000, Tsavaris et al. 2002, Carson et al. 2004). Doxorubicin and 5-fluorouracil are also thought to modulate the activity of APCs. The ability of multiple chemotherapeutic agents to influence the adaptive immune response in positive and/or negative ways is a strong argument for the careful pharmacodynamic analysis of cancer vaccines and chemotherapy in clinically relevant preclinical models rather than simply adding them to a treatment regimen considered to be the standard of care.

Cancer vaccines and therapeutic monoclonal antibodies

Therapeutic monoclonal antibodies can be used to modify tumor cell biology and recapitulate the humoral immune response, or to target critical immunologic checkpoints controlling antitumor immunity. There are emerging data to suggest that humoral immunity may play a more important role in antitumor immunity than previously appreciated (Vasovic et al. 1997, Dyall et al. 1999, Wu et al. 2000, Reilly et al. 2001a,b, Yang et al. 2001). Multiple mechanisms for potential synergy between therapeutic monoclonal antibodies and vaccines exist. For example, trastuzumab, the humanized monoclonal antibody specific for HER-2/neu, can recruit innate immune effectors by orchestrating the development of ADCC (Clynes et al. 2000). Secondly, trastuzumab enhances the lytic activity of MHC Class I restricted HER-2/neu-specific CTL against HER-2/neu-overexpressing breast and ovarian tumor cells (zum Buschenfelde et al. 2002), probably by enhancing the ubiquitination and degradation of internalized HER-2/neu molecules to augment antigen presentation (Klapper et al. 2000, Castilleja et al. 2001). Thirdly, trastuzumab both exerts...
a direct antitumor effect by inhibiting growth-promoting signaling pathways, and renders the tumor cells more susceptible to apoptosis. Supporting the concept that antigen-specific humoral and cellular immune effectors act in concert, we have demonstrated that the adoptive transfer of HER-2/neu-specific antibody with HER-2/neu-specific cytotoxic T lymphocytes results in a more robust antitumor effect than the adoptive transfer of either alone in SCID mice (Reilly et al. 2001b). Extending these studies to tolerized neu mice, we have shown that the combination of HER-2/neu-specific monoclonal antibodies and an HER-2/neu-directed GM-CSF-secreting cellular vaccine that delivers HER-2/neu exerts a more potent antitumor effect than either alone, resulting in the cure of about 40% of mice with pre-established HER-2/neu-expressing tumors (Wolpoe et al. 2003). These studies showed that the addition of HER-2/neu-specific monoclonal antibodies to vaccination can augment the numbers of vaccine-induced HER-2/neu CD8+ T cells as measured by ELISPOT. Other investigators have demonstrated that HER-2/neu-specific IgG induced by peptide vaccination can inhibit tumor cell growth and mediate ADCC (Jasinska et al. 2003). These observations together provide a compelling argument for testing HER-2/neu-targeted vaccines in combination with trastuzumab in patients with HER-2/neu-overexpressing high-risk primary or metastatic breast cancer.

Therapeutic monoclonal antibodies can also be used to target immunologic checkpoints. To date, a monoclonal antibody specific for CTLA-4 has been tested in two published studies. Hodi et al. (2003) administered a single dose of the antibody to five patients with metastatic melanoma or ovarian carcinoma previously immunized with GM-CSF-secreting autologous tumor cells; four additional melanoma patients also received the antibody, three who were vaccinated with autologous dendritic cells presenting the melanoma antigens gp100 and MART-1, and one who was vaccinated with modified gp100 peptide and high-dose IL-2. No clinically significant toxicities were observed, but three melanoma patients previously immunized with GM-CSF-secreting vaccines developed extensive tumor necrosis with immune infiltrates, and two patients with ovarian cancer demonstrated stable to declining levels of the tumor marker cancer antigen-125 (CA-125). Melanoma patients immunized with other types of vaccines demonstrated less vigorous antitumor responses. Phan et al. (2003) treated 14 patients with metastatic melanoma with serial infusions of the CTLA-4-specific antibody in conjunction with gp100-specific peptide vaccination. In this study, six individuals (43%) developed Grade III autoimmunity (dermatitis, enterocolitis, hepatitis, hypophysitis), and three (21%) developed objective evidence of tumor regression. Further study with this CTLA-4 blocking antibody is clearly warranted.

Conclusions and future directions

Early clinical trials of various breast cancer vaccine formulations have established a good safety record to date, and have provided preliminary evidence of potentially clinically relevant bioactivity. They have also highlighted the inherent difficulties in the clinical development of breast cancer vaccines. These include the hurdles posed by established disease burdens and firmly entrenched mechanisms of immune tolerance, and the difficulty of insinuating vaccine therapy into the management of a cancer for which we are fortunate to have a variety of therapies with some established level of efficacy. Elucidating the interactions between established breast cancer therapies and experimental vaccines should facilitate their rational integration into combinatorial treatment regimens with a higher likelihood of clinical efficacy. Moreover, dissecting the most relevant mechanisms of tumor-specific immune tolerance and immune evasion should result in the development of novel targeted therapeutics that can circumvent resistance to immunotherapy by abrogating immune tolerance in a targeted fashion. CD4+CD25+ T regulatory cells have been definitely established as a potent target for manipulation to maximize the response to tumor vaccines. An emerging area for drug development targets dendritic cell biology itself, specifically toll-like receptors (Zuany-Amorim et al. 2002) and co-stimulatory signaling pathways (Table 1). Each of these areas offers opportunities for developing combinatorial vaccination regimens. Two such immunomodulatory drugs are already under active investigation in combination with cancer vaccines in early clinical trials. These include ONTAK (Ligand Pharmaceuticals Inc., San Diego, CA, USA a targeted therapeutic that delivers diphtheria toxin to CD25+ T cells), used to abrogate the negative influence of CD4+CD25+ T regulatory cells (Vieweg et al. 2004), and CTLA-4-specific monoclonal antibodies, used to block the negative signaling pathways that limit tumor-specific T cell activation in response to immunization (Hodi et al. 2003, Phan et al. 2003). With the increasing number of regulatory pathways being identified, new drugs are sure to follow. These areas represent the future of breast cancer immunotherapy. Careful consideration of all of these issues in the design of future trials should hasten the clinical development of breast cancer vaccines,
ultimately facilitating their incorporation into standard clinical practice.

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