Review

Clinical Studies of Vaccines Targeting Breast Cancer

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Abstract

Many clinical studies have been undertaken to assess the therapeutic potential of vaccination and have included a large variety of cancer immunogens. Most of these studies involved patients with metastatic cancer, which is characterized by the most aggressive malignant cells, the longest-lasting disease, and the failure of all standard cytotoxic treatments. The presence of tumor over long periods and the toxicity of previous treatments tend to negatively affect immune responsiveness to tumor antigens presented by the vaccine. In this review, we analyze the ability of past and current vaccine therapies to induce clinical responses in breast cancer. To date, clinical responses have been observed by using vaccines targeting HER-2/neu protein, human telomerase reverse transcriptase, carcinoembryonic antigen, and carbohydrate antigen given after stem cell rescue. The review concludes with a discussion of possible future directions for vaccine development and applications.

Introduction

The finding that immunological manipulation could cause regression of established invasive human cancer came to light after IL-2 was given to patients with metastatic renal cancer or melanoma (1). Although IL-2 has no direct effects on cancer cells, it activates NK cells and T cells. Because T cells expressing antitumor effects should already have been primed by exposure to Ag, significant efforts have been devoted to identifying Ags that are recognized by human T lymphocytes (2). To that end, advances in the molecular characterization of human tumors have led to the identification of tumor Ags that can be used for vaccination purposes (3–6). Successful induction of Ag-specific CTLs and Abs against tumor Ags has been reported after vaccination with materials derived from tumors, transfection of tumor cells with cDNA encoding various cytokines, and the use of dendritic cells that had been pulsed with tumor lysates or tumor peptides; however, most of these studies were Phase I or Phase II clinical trials.

Cancer vaccines represent a nontoxic therapeutic modality potentially capable of inducing antitumor immune responses in patients with primary tumors and in those with metastases. In addition, induction of strong immunity by cancer vaccines is expected to lead to the establishment of immune memory, thereby preventing tumor recurrence. The nature of the immunological variables that should be elicited by the vaccine and are associated with tumor regression is unclear. In this review, we discuss reported responses to vaccines, in terms of toxicity, immunogenicity, immunity duration, and clinical effects, in patients with breast cancer. Vaccine studies are discussed in the following order: tumor cell-based vaccines; tumor Ag protein- or peptide-based vaccines; anticalcineurin-Ag vaccines; and anti-idiotypic vaccines. Whenever possible, we present findings on T-cell (cellular) responses first, followed by findings on B-cell (humoral) responses.

Tumor Cell-based Vaccines

Earlier studies of human cancer vaccination were based on the hypothesis that because autologous or allogeneic tumor cells express many TAAs, presentation of those TAAs in the presence of a strong adjuvant (e.g., BCG, influenza virus, avian influenza virus, or NDV) or cytokines known to promote T- and B-cell proliferation will activate innate immunity that will be sufficient to maintain an immune response against tumor, and that that immune response will lead to a clinical response. Potential concerns arose from the unknown amounts of tumor Ag that were administered, the unknown immunodominance of the tumor Ag, and the safety of using tumor DNA. Freedman et al. (7, 8) used viral oncolysates to treat advanced ovarian cancer and high-risk untreated squamous cell carcinoma of the uterine cervix-stimulating factor; DTH, delayed-type hypersensitivity; CEA, carcinoembryonic antigen; hTERT, human telomerase reverse transcriptase; TCR, T-cell receptor; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cell; TF, Thomsen-Friedenreich antigen; Lea, Lewisb pentasaccharide; OSM, ovine submaxillary mucin; Adv, adenovirus; rVV, recombinant vaccinia virus; rF, recombinant fowlpox virus; T9, T helper; ALVAC, recombinant canarypox (avian-pox) virus.

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4 The abbreviations used are: IL, interleukin; NK, natural killer; Ag, antigen; Ab, antibody; TAA, tumor-associated antigen; BCG, Bacillus Calmette-Guérin; NDV, Newcastle disease virus; APC, antigen-presenting cell; HLA, human leukocyte antigen; GM-CSF, granulocyte macrophage colony-stimulating factor; DTH, delayed-type hypersensitivity; CEA, carcinoembryonic antigen; hTERT, human telomerase reverse transcriptase; TCR, T-cell receptor; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cell; TF, Thomsen-Friedenreich antigen; Lea, Lewisb pentasaccharide; OSM, ovine submaxillary mucin; Adv, adenovirus; rVV, recombinant vaccinia virus; rF, recombinant fowlpox virus; T9, T helper; ALVAC, recombinant canarypox (avian-pox) virus.

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cervix. These oncolysates were prepared from lysates of cultured ovarian and cervical lines infected with the influenza A/PR8/34 virus. The encouraging results (namely, the disappearance of ascites in several patients, the disappearance of pleural effusions in one patient, and the shrinkage of tumor masses in two patients without ascites) provided strong support for the hypothesis that active immunotherapy with cancer cells given in combination with a viral helper or immunomodulator could induce clinical responses.

**Autologous Tumor Vaccines.** Ahlert et al. (9) investigated the efficacy of a purified, irradiated autologous tumor cell vaccine-NDV vaccine in three independent cohorts of patients who were vaccinated between 1991 and 1995 in an attempt to induce a DTH response. The vaccine consisted of tumor cells infected with NDV, the rationale being that NDV induces strong immune responses, it will potentiate the local cellular response to tumor cells through secretion of cytokines, activation of APCs, or both. The authors vaccinated 63 patients with primary breast cancer, 27 patients with previously treated metastatic breast cancer, and 31 patients with previously treated metastatic ovarian cancer; tumor cell number and viability were compared retrospectively with patient survival. Trends toward improved survival and disease-free survival were associated with use of a vaccine that had minimal dead cell contamination and maximal viability, implying the possibility that such a vaccine could be clinically effective in patients with breast or ovarian cancer.

One approach designed to provide continuous local production of a desired cytokine is gene modification of the tumor cells themselves (10, 11). This approach is based on evidence that local cytokine secretion can activate T and NK cells as well as inducing granulocytic inflammatory responses against tumors. Elder et al. (10) used IL-4 gene-modified fibroblasts in combination with irradiated autologous tumor cells as an alternative to cytokine-gene transduction of autologous tumor cells. In that study, isolated skin fibroblasts grown in primary cultures were infected with different cytokine-gene transduction of autologous tumor cells. In that study, isolated skin fibroblasts grown in primary cultures were transduced with a retroviral vector containing the human IL-4 gene and the Neo gene as a selection marker. Of 33 transduced fibroblast cultures obtained from the study participants, 21 produced at least 1000 units of IL-4/24 h/10⁶ cells for 3 weeks. Similar results were obtained with a retroviral vector encoding IL-2. The clinical outcome of this strategy has yet to be reported.

**Allogeneic Vaccines.** Wiseman (12) reported a 10-year follow-up analysis of 13 patients with inflammatory breast carcinoma treated with surgery, chemotherapy, and allogeneic tumor cell/BCG immunotherapy. In that study, patients had been given chemotherapy followed by surgery and continued chemotherapy for 24 months, after which radiation was delivered to the chest wall and regional nodal basins. All patients were given 10⁷ irradiated viable tumor cells pooled from three cultured cell lines admixed with 10⁷ Connaught BCG organisms. At 10 years of follow-up, 4 of the 13 patients (31%) were alive and free of disease. The results also indicated an apparent plateau in the survival curve at about 5 years. The authors suggested that a multimodality approach is not only feasible in such high-risk populations but may also lead to long-term survival.

In two other more recent studies, HLA-A2+ patients with stage IV breast cancer were immunized with an allogeneic breast cancer cell line (MDA-MB-231) that expresses CD80 admixed with graded doses of GM-CSF or BCG as adjuvants (13, 14). A few patients displayed low-level responses to an Ag expressed by the tumor, and several had more substantial DTH responses to CD80-modified tumor cells as compared with unmodified controls, but overall, no significant differences in the extent of DTH reactions were noted. The clinical outcome of this study has yet to be reported.

**Mixed Autologous/Allogeneic Tumor Vaccines.** Jiang et al. (14) reported using a multi-Ag vaccine that included Ag from autologous breast cancer cells, the allogeneic breast cell line MCF-7, and the TAAs CA15-3, CEA, and CA125, plus low doses of GM-CSF and IL-2. In that study, 42 patients with breast cancer (4 patients with stage II disease, 14 patients with stage III disease, and 24 patients with stage IV disease) were given s.c. vaccinations in the first, second, third, seventh, eleventh, and fifteenth weeks of the study. All patients also underwent mastectomy with axillary node dissection before vaccination. Disease improvement was observed in two patients (12%). One of those patients had multiple liver metastases at first vaccination; after the sixth vaccination, computed tomography scanning showed that all of the liver metastases were smaller, and some had disappeared. By 8 months later, however, computed tomography scans showed progressive liver and lung metastases. The other patient had stage IV breast cancer with metastases to the L5 vertebra and skull; after her sixth vaccination, a bone scan showed shrunken and healing metastases. By 14 months later, however, a bone scan showed progression of the bone metastases. The vaccine induced a significant increase in in vitro lymphocyte proliferative responses to autologous tumor Ag, CA 15-3, CEA, and CA125, but not to the allogeneic tumor Ag. Vaccination did not significantly reduce the levels of the serum tumor markers CA 15-3, CEA, and CA125.

**Breast Cancer Antigen-specific Vaccines**

The discovery of T-cell recognition and lysis of human tumors subsequently led to the identification of specific onco-proteins or mutated oncoengens (e.g., MUC-1, -HER-2, CEA, and p53). Many of these tumor antigens are found on normal tissues but are recognized by the immune system due to their overexpression. Some proteins are tumor specific, but others, such as the universal tumor Ag hTERT, are broadly expressed by most tumor cells. These antigens are discussed below.

**Vaccines Using Proteins and Peptides from Tumor Ag as Immunogens.** Mucin (MUC-1) is present on normal ductal epithelium, where it lacks the overexpression and underglycosylation typical of tumor tissue (15). Tumor MUC-1 is recognized by CTLs in either a MHC-unrestricted manner or a MHC-restricted manner (16–19). A protein containing approximately 20 tandem repeats of the conserved unglycosylated 20-amino acid core sequence of the tumor MUC-1 protein is believed to have adequate affinity for binding to TCRs.

Early preclinical studies (20–23) using tumor cells that expressed MUC-1 protein or peptide Ag concluded that MUC-1 could induce a humoral response without inducing cellular responses. With the goal of inducing cellular responses, Goydos et al. (24) tested a 105-amino acid synthetic MUC-1 peptide
with five repeated immunodominant epitopes. In that study, 63 patients with adenocarcinoma of the breast (n = 9), colon (n = 30), or pancreas (n = 24) were vaccinated with 100 μg of the synthetic MUC-1 peptide mixed with BCG three times at 3-week intervals. All patients underwent DTH testing with the 105-mer and shorter mucin peptides 48 h to 1 week before the first vaccination. Three patients showed a strong DTH response to the full-length peptide at the injection site. Biopsy samples showed intense T-cell infiltration in 37 patients and lesser infiltration in 7 patients. Seven of 22 patients had 2-fold to 4-fold increases in mucin-specific CTL precursors after vaccination as compared with before. Whether mucin-specific Abs were induced was unclear. Three patients (two patients with colon cancer and one patient with pancreatic cancer) showed disease stabilization after the vaccination.

An alternative approach was used by Reddish et al. (25), who gave 16 patients with metastatic breast carcinoma a vaccine consisting of 5 μg of the 16-amino acid MUC-1 peptide BP-16 (GVTSAPDTPAPGSTA) coupled to KLH and DETOX as adjuvants. Low Ag concentrations were hypothesized to activate a Th1 cytokine response; thus the low concentration of MUC-1 was expected to activate cellular responses. Patients underwent four vaccinations, with low-dose cyclophosphamide (300 mg/m²) given before the first vaccination and after the second vaccination. Four weeks after the fourth vaccination, class I HLA-restricted anti-MUC-1 CTLs were detected in seven patients; five of these seven patients also had high anti-MUC-1 IgG titers. Class I-restricted anti-MUC-1 lytic activity was also observed after a single in vitro stimulation with a synthetic MUC-1 peptide. The clinical effects of inducing this level of MUC-1 immunity have not been reported. In most patients, development of MUC-1-specific class I-restricted CTL precursors correlated with IgG responses, suggesting that Ag-specific CTL precursors were present and circulating in the blood, but these cells were not differentiated effectors. The apparent difficulty in generating CTL effectors in vivo may have been due to the induction of T-cell anergy by tumor-derived MUC-1 (26). In an attempt to optimize MUC-1 presentation by APCs, several studies have used the mannose receptor on APCs for binding molecules that have been linked to mannose. One group used the mannam-MUC-1 fusion protein, consisting of five repeats of the 20-amino acid MUC-1 variable number of terminal repeats linked to mann, for experimental immunotherapy in mice and in humans. Use of the oxidized form of the fusion protein typically produced high numbers of CTL precursors (27–30). Vaccination of 25 patients with metastatic breast (n = 8), colorectal (n = 16), or stomach carcinoma (n = 1) with escalating doses of oxidized fusion protein (10–500 μg) led to high titers of high-affinity anti-MUC-1, IgG1 Ab (1:320 to 1:20,480) in 13 patients after four to eight immunizations (31). The levels of Ab directly correlated with the amount of immunogen given. The amounts of Ab produced to MUC-1 in that study were much higher than those in previous reports involving MUC-1 carbohydrate immunogens (32–35), but the Abs in the human fusion-protein study were against peptides (31). Interestingly, the resulting Abs were not directed against the sequence APDTR, as might have been expected from the specificity of the murine antihuman MUC-1 Ab (36). Rather, most of the Abs reacted to the adjacent epitopes STAPPAHG and PAPGSTAP. MUC-1-specific T-cell proliferation was observed in 4 of 15 patients, and MHC-I-restricted CTL responses were noted in 2 of 10 patients (31). Clearly, the oxidized mannam-MUC-1 fusion protein was immunogenic in humans, particularly for the formation of Ab and to a lesser extent for cellular responses.

To test the hypothesis that tolerance to TAAs can be broken by using vaccines consisting of Ag coupled with KLH plus potent adjuvants, nine high-risk breast cancer patients with no evidence of disease underwent immunization with MUC-1-KLH plus QS-21 (37). The vaccine in that study contained 100 μg each of MUC-1 and QS-21. In another study (38), eight patients with documented metastases to the lung (n = 1), the chest wall (n = 4), and the supraclavicular lymph nodes (n = 3) and one patient with mildly elevated CEA levels were given the same vaccine. In that study, high IgM and IgG titers against MUC-1 were detected, and the IgG titers remained elevated for 106–137 weeks or more after the first vaccination. In seven patients, binding of IgM on MCF-7 cells was noted, indicating that these Abs bound to MUC-1 naturally expressed on tumor cells; however, binding of IgG was minimal. Abs from six patients bound primarily to the APDTRPA epitope of the MUC-1. No evidence of T-cell activation or clinical responses was found. A more recent study by the same group reexamined T-cell activation and differentiation by MUC-1-based vaccines (39). In that study, six patients with breast cancer were vaccinated four times with a 106-amino acid MUC-1 peptide with KLH and QS-21. The T-cell response to MUC-1 was minimal and inconsistent compared with the response to KLH, regardless of whether cells were stimulated in vitro and cultured in IL-2 (39). No evidence of an autoimmune reaction was found.

HLA-A2-restricted T-cell epitopes derived from MUC-1 have been identified. M1.1 (STTPPVHNV) is derived from the variable number of terminal repeats domain of MUC-1, whereas M1.2 (LLLLTVLTV) is located in the leader sequence (19). Because previous vaccine studies in which different forms of MUC-1 were used showed a predominantly humoral (Ab) response in humans, Brossart et al. attempted to direct the response toward epitope-specific CTLs by using vaccination with dendritic cells pulsed with peptides corresponding to HLA-A2-restricted CTL epitopes (40). In that study, dendritic cells were obtained from adherent PBMCs after being cultured with GM-CSF and IL-4 followed by tumor necrosis factor α; the phenotype of those cells was consistent with that of mature dendritic cells. In preclinical studies, CTLs were generated from several healthy donors via primary in vitro stimulation by MUC-1 peptide-pulsed dendritic cells together with the pan-HLA-DR-binding T-helper epitope “PADRE” (19, 41). These CTLs lysed tumor cells that expressed MUC-1 in an HLA-A2-restricted, Ag-specific fashion.

In another Phase I study, the feasibility and efficacy of vaccination with mature dendritic cells that had been pulsed with HER-2/neu- and MUC-1-peptides were tested in heavily pretreated, HLA-A2+ patients with refractory metastatic breast (n = 7) or ovarian cancer (n = 3). Tumors in all patients expressed HER-2/neu or MUC-1. The Ags used were E75 (369–377):KIFGSLAFL and GP2:HIASVGIL, both HLA-A2-binding peptides from HER-2; and the MUC-1 peptides M1.1 and M1.2 (19, 42, 43). In 5 of these 10 patients, peptide-specific CTLs were detected in the PBMCs even after high-dose chem-
otherapy. The major in vivo CTL response induced by the E75 and M1.2 peptides lasted for more than 6 months. Interestingly, in one patient vaccinated with MUC-1 peptides, CEA- and MAGE-3 peptide-specific T-cell responses also were detected after several vaccinations. In another patient immunized with HER-2/neu peptides, MUC-1-specific T lymphocytes were activated. These findings suggested that in vivo Ag spreading may take place after vaccination with peptide-pulsed dendritic cells. One patient with metastatic breast cancer who was given MUC-1-pulsed dendritic cells experienced regression of several s.c. lesions. The responses observed in this study cannot be considered proof that the vaccination had induced the responses because the patient underwent concurrent oophorectomy.

HER-2/neu. In addition to being overexpressed by some cancer cells, HER-2 is widely expressed in normal epithelial tissues, including breast tissue, in human fetuses and adults. The HER-2 gene is amplified in 30% of primary human breast and ovarian cancer cases (44). The HER-2 protein has also been found to be immunogenic in some patients with breast or ovarian cancer, with CD4+ and CD8+ immunogenic peptides having been identified; thus HER-2 is a potential target for vaccines (45–49).

In one study, Zaks et al. (50) vaccinated patients with metastatic breast (n = 1), ovarian (n = 2), or colorectal (n = 1) cancer with 1 mg of E75 in incomplete Freund’s adjuvant every 3 weeks. In three of the four patients, peptide-specific CTLs were easily detected in the blood after one immunization. Although these CTLs did not specifically lyse HLA-A2+, HER-2+ tumor cells, some produced more IFN-γ in response to HER-2− than to HER-2+ tumors. No clinical responses were observed in that study. In another Phase I study, Knutson et al. (51) tested a vaccine containing three HER-2 peptides, with GM-CSF as an adjuvant, in 60 patients with stage III or IV breast (n = 57), ovarian (n = 1), or non-small cell lung cancer (n = 2). Patients were vaccinated once a month for 6 months with one of three vaccine formulations comprising potentially class II-binding peptides derived from the extracellular (n = 28) or intracellular domains of the HER-2 molecule (n = 23) or, if the patients were HLA-A2+, with peptides containing CTL epitopes (n = 19). In an interim analysis of the first 22 patients to receive all six vaccinations, 21 (95%) showed T-cell proliferative responses to one or more of the immunizing peptides, and 16 (73%) showed responses to the HER-2 protein. In another series of studies, 18 patients with breast cancer (4 with stage III disease and 14 with stage IV disease) were given monthly vaccinations of three 15-amino acid HER-2 peptides (369–384, 688–703, and 971–984) that contained the nested CTL epitopes 369–377, 688–696, and 971–979 (42, 47, 52). The goal of these studies was to overcome the problems associated with using class I epitopes alone, most of which are related to Ag instability or aggregation because of the short length of the peptide. CD4+ cell responses may help the survival of CD8+ cells, particularly when the initial CTL frequency is low (53). In that study, the mean numbers of peptide-specific CTL precursors increased in most patients, and the peptide-specific CTLs lysed tumor cells. The CD4+ and CD8+ cell responses were long-lasting and remained detectable for more than 1 year after the final vaccination in some patients. Clinical responses in these patients have yet to be described.

Murray et al. (54, 55) used a vaccine containing E75 plus GM-CSF for a Phase I trial of 14 patients with metastatic breast (n = 13) or ovarian (n = 1) cancer. These patients were vaccinated with escalating doses (500–1000 μg) of E75 mixed with 250 μg of GM-CSF. No grade 3 toxic reactions to the vaccine were noted. Of eight patients tested for CTL induction, four had a CTL response after in vitro stimulation with autologous dendritic cells that had not been pulsed with peptide, consistent with the presence of activated/memory cells ex vivo. Four patients also had a E75-specific CTL response after in vitro stimulation with E75. E75-specific CTLs from three patients specifically recognized E75 on indicator tumors, as demonstrated by cold target inhibition of tumor lysis. E75-specific tumor-lytic CTLs were present in some patients for more than 1 year after vaccination. A trend was apparent that patients with positive DTH, proliferation, or both had longer time to progression (P = 0.06; Ref. 55).

Rejection of an established cancer is a difficult, if not impossible, task for the immune system. To properly assess the effectiveness of a vaccine or vaccine strategy, trials should target patients with nonmetastatic disease. Peoples et al. have begun a preventive trial of the E75/GM-CSF vaccine discussed above for patients with breast or prostate cancer who have been treated and are currently disease free but at high risk of recurrence (48). Preliminary results from the first 27 vaccinated patients revealed no significant toxicity; dosing and scheduling issues continue to be evaluated. All of the patients who completed the vaccination series showed an immunological response to the HER2/neu peptide, as measured by dimer staining and flow cytometry. This documentation of clonal expansion has correlated well with functional assays. The presence of E75-specific CD8+ T cells has also been demonstrated in 57% of patients 6 months after completion of the inoculation series. Some evidence of intra-antigenic epitope spreading has been noted; however, clonal exhaustion was also seen after repeated vaccination and prompted a change in the vaccination schedule (48).

CEA. Because wild-type tumor Ags (CTL epitopes) seem to be weak immunogens, Engleman et al. developed a novel dendritic cell-peptide vaccine and used it in a Phase I clinical trial of metastatic or recurrent colon cancer or small cell lung cancer in which serum CEA levels were abnormal or rising (56). In that study, dendritic cells (which are present in small numbers in the peripheral blood) were first expanded with Flt3 ligand in vivo, after which the patients were immunized with KLH and dendritic cells that had been pulsed with peptide from the CEA CAP peptide or a CAP1–6D (YLSGANLNL) peptide in which the asparagine (N) was replaced with glutamic acid (D). After immunization, native CAP1 and altered CAP1–6D peptide-specific and tumor-reactive CTLs were observed by CTL assay and tetramer staining. One patient with progressive metastatic colorectal cancer had complete resolution of pulmonary metastases and malignant pleural effusions at 4 months after vaccination, and 2 of 12 patients showed disease stabilization. The occurrence of clinical response correlated with the expansion of CD8 tetramer+ T cells, confirming the role of CD8 T cells in the antitumor responses.

hTERT. hTERT is a common, nearly universal, tumor antigen and is expressed in more than 85% of all human cancers.
Breast Cancer Vaccines

with the melanoma differentiation antigen GM2 and results of randomized Phase III trials in which immunization in this strategy. Support for these approaches is provided by developing responses to carbohydrate Ag is considered an advantage because T cells and NK cells can recognize and destroy hTERT+ cancer cells (59). The first Phase I trial of this approach, in which the vaccine consists of peptide-pulsed dendritic cells, is under way at the Dana-Farber Cancer Institute for HLA-A2+ patients with advanced cancer. A second Phase I trial for patients with metastatic prostate cancer in which hTERT mRNA-transfected autologous dendritic cells are being used as the vaccine is under way at Duke University (59). A third Phase I trial for patients with other forms of metastatic, HLA-A2+ cancer involves giving a vaccine consisting of hTERT peptide in incomplete Freund’s adjuvant; IL-2 is given only to patients with melanoma. Further information on these trials is available online.5

p53. p53 is the most commonly mutated gene in human cancers and is mutated in about 20% of breast cancers (61). In many tumor cells, mutations in p53 result in the accumulation of mutated p53 products. Because most p53 mutations involve the alteration of a single amino acid, it follows that most of the p53 epitopes presented to immune cells by tumor would be wild type in sequence (62). No clinical studies of gene therapy with p53 for breast cancer have been reported, although studies of p53 immunogenetic therapy for other human cancers are ongoing [described in the section entitled “Vaccines in Which Tumor Ag Is Expressed by Recombinant Viral Vectors” (63, 64)]. Advances in gene therapy technology and replicable clinical results from such studies may extend the use of p53 as a breast cancer treatment.

Vaccines Inducing Humoral and Cellular Responses against Carbohydrate Antigens. Breast cancer vaccines in which Tn, TF, sialyl-Tn, LeX, or Globo H Ag are used as immunogens have been developed based on the hypothesis that the de novo expression of carbohydrate Ag can induce Ab responses, which then induce antitumor responses, either by Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity or by interfering with receptor-mediated signaling, adhesion, and metastasis. The fact that T cells and NK cells develop responses to carbohydrate Ag is considered an advantage in this strategy. Support for these approaches is provided by results of randomized Phase III trials in which immunization with the melanoma differentiation antigen GM2 and BCG was shown to reduce the risk of relapse in patients with stage III melanoma who were free of disease after surgical resection and had no preexisting anti-GM2 antibodies (65). Other, more recent studies of melanoma (66–68) used GM2 and GD3 gangliosides coupled to KLH and QS-21 as adjuvant instead of BCG, with the aim of enhancing the production of ganglioside-specific IgG and IgM. The results reported to date suggested that such vaccines seem to be the most promising in inducing immunological responses if they were used in combination with cyclophosphamide and stem cell rescue.

Sialyl-Tn. Carcinoma-associated mucins often show incomplete glycosylation (69), possibly because the cells that produce them are deficient in certain glycosyltransferases (70). This incomplete glycosylation leads to the formation of shortened carbohydrate side chains and exposure of the normally cryptic O-linked core carbohydrate determinants such as Tn and TF. The Tn antigen is a short carbohydrate structure that is O-linked to serine or threonine on the protein backbone (Gal-N-Acaol→O-Ser/Thr). This structure can be extended with a galactose residue to form the TF Ag (Galβ1–3Gal-N-Acaol→O-Ser/Thr), or it can be substituted with sialic acid, resulting in the sialyl-Tn Ag (Fig. 1). Thus the Tn, TF, and sialyl-Tn Ag represent the initial, most immature glycosylation products of serine and threonine of the protein core and are masked in normal tissue because of sialylation or chain elongation and branching by the addition of other sugar residues, such

5 www.clinicaltrials.gov.
as blood group determinants (71). Sialyl-Tn is a core-region carbohydrate Ag that is formed by the premature 2–6 sialylation of N-acetylgalactosamine, the expression of which has been associated with some human malignancies. All three epitopes (TF, Tn, and sialyl-Tn) are strongly expressed on cancer cells and may be associated with disease progression and metastasis.

In a study by MacLean et al. (72), a vaccine was prepared that consisted of the synthetic form of the immunodominant disaccharides (β-Gal1–3α-GalNAc) of the TF Ag conjugated to KLH (TFα-KLH) plus DETOX. A classic anti-Tα response, in which an initial increase in IgM titers was followed by an increase in IgG and IgA, was observed in patients with ovarian cancer after s.c. vaccination injection (72). Patients with breast cancer vaccinated with low-dose (100 μg) or high-dose (500 mg/m²) TFα-KLH plus a single low dose of cyclophosphamide (200 mg/m², i.v.) developed DTH reactions at the vaccination sites (73). In another study by the same group, MacLean et al. (74) used a synthetic sialyl-Tn hapten conjugated to KLH with DETOX as an adjuvant to vaccinate patients with breast cancer. All patients had increases in IgM and IgG Ab titers specific for the synthetic sialyl-Tn, and the IgM titers were higher among patients given cyclophosphamide. There were no significant increases in antibody titers to TFα-KLH. IgM and IgG Abs against OSM, which carries multiple repeating natural sialyl-Tn determinants, were also detected. However, no correlation was found between the Ab titers to synthetic sialyl-Tn or an OSM-sialyl-Tn conjugate, complement-mediated tumor cell cytotoxicity, and the clinical responses observed. Clinical responses were noted in one patient, whose pulmonary metastases remained stable for 15 months after entry in the program, and in five patients who were alive after 12 or more months.

To further clarify the role of cyclophosphamide in sialyl-Tn vaccination, MacLean et al. (74) also immunized patients with breast cancer with THERATOPE, a sialyl-Tn-KLH conjugate, and DETOX after they had been given a low dose of cyclophosphamide (300 mg/m² i.v. once on day 30 or 50 mg/m² p.o. daily) in parallel with patients who were not given cyclophosphamide. The anti-sialyl-Tn and anti-OSM Ab titers were higher among the patients given i.v. cyclophosphamide. Patients given i.v. cyclophosphamide also lived longer (projected median survival of 19.7 months versus actual median survival of 12.6 months; P = 0.0176) than did those given oral or no cyclophosphamide. Moreover, a correlation was evident between vaccine-induced Ab response and clinical course after immunization with sialyl-Tn-KLH + DETOX (74). In that study, the nine patients with breast cancer who showed a IgM Ab response to OSM that exceeded the median survived significantly longer than did the 15 patients who demonstrated a lower-than-median response. No correlation was found between anti-KLH Ab titers and survival, demonstrating the specificity of the association of the anti-OSM Ab levels with survival. Finally, patients given low-dose i.v. cyclophosphamide before vaccination survived longer and had higher anti-OSM Ab titers than did patients given low-dose oral cyclophosphamide before vaccination.

In another study, Sandmaier et al. (75) gave THERATOPE to 33 patients with high-risk stage II-III or stage IV breast cancer 30 to 151 days after they had completed high-dose chemotherapy followed by stem cell rescue. The ration-
Anti-idiotypic Vaccines

Idiotype manipulation is the major tumor-specific active approach that does not use tumor-derived material to induce antitumor immunity (reviewed in Ref. 80). Anti-idiotypic responses have been involved in the induction of clinical responses in colorectal cancer. Several idiotype cascades have been generated by using a T-cell leukemia-associated Ag, CEA, GD2, and human milk fat globule membrane Ag as immunogens (80–82). Bhattacharya-Chatterjee and others (82, 83) completed a Phase Ib vaccine trial in which patients with metastatic breast cancer were given s.c. injections of the anti-idiotype Ab designated 11D10; 11D10 (Ab2) was raised against the antigenic milk fat globule membrane monoclonal antibody MC-10 (Ab1). In that trial, 33 patients were randomized to receive 1, 2, 4, or 8 mg of 11D10 every other week for a total of 8 weeks and then monthly until disease progression. Of the 19 patients who were given more than four immunizations, 16 had an anti-anti-idiotype (Ab3) response to human milk fat globule membrane Ag that inhibited the binding of Ab2 to Ab1. The Ab3 also induced in vitro idiotype-specific T-cell proliferation, suggesting that 11D10 can induce both humoral and cellular immune responses. Like the response to sialyl-Tn in other studies, a rapid immune response was observed when vaccination followed autologous stem cell transplantation. Interestingly, the patients with the most vigorous humoral and cellular response had significant improvements in progression-free survival (83).

Vaccines in Which Tumor Ag Is Expressed by Recombinant Viral Vectors. The finding that viral infections lead to the presentation of viral peptides in association with MHC class I and class II Ag on the surface of infected cells has led to the design of strategies in which viruses are used as the immunization vehicles (vectors). Studies in melanoma reported that use of Adv-based vectors is equivalent to use of peptides or proteins in terms of inducing functional tumor-specific CTLs (84). One recent approach involved modifying autologous tumor cells to overexpress IL-7 and GM-CSF together with double stem-loop immunomodulating oligodeoxyribonucleotides as a Tr1-promoting modulator; preliminary reports show clinical responses (one complete response, one partial response, and two cases of stable disease) among the 10 patients treated (85). Two completed studies in which rVV or the ALVAC recombinant canarypoxvirus-based vector system were used are discussed further in the remainder of this section. Additional information on the use of the ALVAC is presented in Ref. 86.

CEA. CEA was one of the first tumor markers to be identified and characterized (reviewed in Ref. 80). Use of CEA as an immunological target has been the focus of several studies (87–89) that identified an immunodominant epitope, CAP-1 (YLSGADLNL), and established that the corresponding peptide presented on dendritic cells can activate CTLs from both healthy donors and cancer patients. A rVV has been developed that contained the CEA gene in its genome and expressed its gene product in infected cells; another vector consists of ALVAC expressing the human CEA gene. When the rVV-CEA was used for priming and the ALVAC-CEA was used as a boost in an experimental model, the resulting CEA-specific T-cell responses were at least four times higher than those achieved by using three vaccinations with ALVAC-CEA alone in a separate study. Multiple-boost vaccinations of ALVAC-CEA further potentiated the CEA-specific T-cell responses (90).

To determine whether the sequence of administration of the rVV-CEA and ALVAC-CEA agents would have an effect on T-cell responses and whether vaccination could produce objective responses in metastatic disease, Marshall et al. (91) treated 18 patients with CEA tumors (two with breast cancer) with rVV-CEA (V) and ALVAC-CEA (A). Eleven patients had visceral metastases, and the other seven had no evidence of metastatic disease; disease stage was not noted. The patients were randomized to receive either one rVV-CEA vaccination followed by three ALVAC-CEA vaccinations (VAAA) or three ALVAC-CEA vaccinations followed by one rVV-CEA vaccination (AAAV). Subsequent ALVAC-CEA vaccinations were given to both groups. Both vaccination schedules were well tolerated by all patients. Immunological monitoring, performed by enzyme-linked immunospotting for IFN-γ production to CAP-1 as indicator, indicated that after four vaccination cycles, the number of CAP-1-specific T-cell precursors was increased in all six patients in the VAAA group as compared with two of the five patients in the AAAV group. When local GM-CSF and IL-2 were administered after vaccination with ALVAC-CEA, the number of CAP-1-specific precursors increased in all six HLA-A27 patients. CEA-specific IgG responses were also induced in 4 of the 18 patients, suggesting that this vaccine strategy was more effective in activating cellular (as opposed to humoral) immunity to CEA and that rVV-CEA was more effective as a primer. The VAAA dose schedule was the more effective of the two. ALVAC-CEA was given up to eight times, with continued increases in the numbers of CAP-1-specific T-cell precursors. However, no objective antitumor responses were observed in any of the patients despite the incidence of measurable CEA-specific T-cell responses. This finding may have been related to the presence of advanced disease, insufficient numbers of T cells to elicit measurable reductions in tumor size, high interstitial pressure that prevented T cells from penetrating the tumors, and variations between the patient groups. A more recent study (92) in which ALVAC-CEA-B7.1 (CD80) was used with or without GM-CSF as an adjuvant reported that strategy induced a CEA-specific T-cell response. Of interest for disease detection protocols, the anti-CEA response was determined in this study by using the agonist CAP-1–6D peptide (YLSGADLNL) as indicator under stringent conditions (i.e., 24 h after stimulation) to rule out in vitro selection/dilution of precursors. Vaccination led to a 2-fold increase in number of CEA-specific precursors in 10 of 12 patients so treated. Another important finding of this study was the observation of disease stabilization in patients treated with ALVAC-CEA-B7.1 alone but not in combination with GM-CSF (92).

A new development in approaches targeting CEA is the introduction of TRICOM (a triad of the costimulatory molecules B7.1, intercellular adhesion molecule 1, and lymphocyte function–associated antigen-3). This construct has been delivered by means of an rVV and a recombinant avipox (rF) vector, with GM-CSF administered as a biological adjuvant with all vaccinations. A diversified prime-booster vaccination approach using these TRICOM-expressing vectors was tested in mice transgenic for the human CEA gene. This CEA-targeted vaccine generated strong CEA-specific host responses in the transgenic.
mice, which resulted in a significant reduction in the number of intestinal tumors and improved overall survival (93). On the basis of these results, Theron Biologics and the United States National Cancer Institute have begun several trials of CEATRICOM. One such trial is a multistage, dose-escalation study to assess the safety and immunological effects of CEATRICOM in up to 42 patients with advanced metastatic colorectal cancer. Subjects will be given rF-CEA-TRICOM alone, rV-CEA-TRICOM followed by booster vaccinations with rF-CEA-TRICOM, or rV-CEA-TRICOM followed by rF-CEA-TRICOM, all with GM-CSF as an adjuvant. The primary measure of immune response will be the level of CEA-specific T cells stimulated by vaccination, with levels of CEA-expressing tumor cells in the blood used as a potential secondary measure of treatment effect. Similar studies in breast cancer have been begun as well.

One such trial is a Phase I/II study of rVV-CEA-TRICOM vaccine given before chemotherapy and a rFV-CEA-TRICOM vaccine given after induction and high-dose chemotherapy with autologous peripheral blood stem cell transplantation for patients with previously untreated metastatic breast cancer. Another is a Phase II randomized pilot study of sequential rV-CEA-TRICOM vaccine, rF-CEA-TRICOM vaccine, and GM-CSF with standard adjuvant chemotherapy for women with high-risk stage II-III breast cancer. The third of these studies is a Phase I study of rF-CEA-TRICOM vaccine with or without GM-CSF or rF-GM-CSF for patients with advanced or metastatic CEA-expressing adenocarcinoma. Further information on these trials is available online.4

rALVAC-p53. Van der Burg et al. (63) performed a Phase I/II dose-escalation study of a recombinant ALVAC vaccine encoding wild-type human p53 in 15 patients with p53+ colorectal cancer. Each group of five patients received three i.v. doses of the vaccine. Both humoral and cellular anti-ALVAC responses were induced in all but one patient, and anti-p53 Ab responses were induced in a fraction of patients. Moreover, p53-specific, IFN-γ-producing T-cell immunity was induced in two of five patients vaccinated with the highest dose of ALVAC-p53 (63).

Kuball et al. (64) performed a Phase I clinical trial of a recombinant replication-defective Adv vector encoding human full-length wild-type p53 (rAdv/hup53) in six HLA-A2+ patients with advanced forms of cancer (three urogenital cancers, two lung cancers, and one malignant Schwannoma). Patients were given two injections (one intradermal and one subcutaneous) twice. The treatment was well tolerated. Transient local erythema at the injection site was common in all patients. Amplification of humoral and cellular anti-Adv response was demonstrated in all patients after vaccination; however, p53-reactive antibodies and HLA-A2-restricted wild-type p53-specific CTLs were not generated, nor were objective tumor responses observed. At 7–16 months of follow-up, only one patient had disease stabilization.

Up to this point, there have been several problems associated with rAd vectors used as vaccines. First, their ability to infect dendritic cells is poor. Moreover, expression of tumor Ag in Adv does not reverse the dominant hierarchies in specific CTL induction. Studies are ongoing in an attempt to modify the Adv fibers so as to increase the infectivity of the virus in APCs.

Perspectives and Future Directions
Development of Novel Antibodies with Antireceptor Functions. The potential of immunological therapies and their promise with regard to cancer treatment have been illustrated recently by experience with the anti HER-2 monoclonal antibody trastuzumab [Herceptin (94, 95)]. Traditionally, antitumor Abs were expected to mediate their effects by Ab-dependent cellular cytotoxicity or complement-dependent cytotoxicity (96, 97). However, binding of surface receptors by Ab may interfere with ligand binding or receptor clustering. Ab may also induce receptor internalization or interfere with receptor reexpression. All of these interactions translate into antitumor effects by interfering with tumor cell metabolism and division. Because of their high specificity, flexibility in design, and potential amplification by Ab-Ab complexes, anti-idiotype Abs hold high promise (98). The therapeutic utility of blocking receptors has been established with Herceptin, which blocks HER-2/neu signaling; blocking effects such as these can be amplified by using inhibitors of tyrosine kinases, alone or together with activators of serine or threonine protein kinases. Because such kinases interfere with tyrosine-phosphorylated receptor recycling, their activation may amplify the effects of the Ab. Moreover, in some tumors, activation of receptors may not translate into increased DNA synthesis and tumor proliferation but rather into cell adhesion and motility. Receptor-blocking Abs may be able to control progression to metastasis by blocking the formation of high-affinity receptors (99–102).

Optimization of Vaccine Components for Selective Activation of Effector and Memory Responses. A cancer vaccine can be designed to induce a Th1 or a Th2 environment but cannot induce both simultaneously. The examples of humoral and cellular immunity selectively activated by changes in carriers and adjuvants discussed earlier in this review illustrate this limitation. IL-2 and IL-12 can be added if the goal of the vaccine is to induce effector CTL responses. Similarly, distinct classes of oligodeoxyribonucleotides containing CpG motifs must be included with the vaccine if the objective is to induce effector responses or to maintain antitumor memory in patients. Development of anti-vector Abs that bind vector-infected cells can be minimized by changing the vector at each vaccination, e.g., from the influenza virus to the Adv. A first attempt in this direction was illustrated by the diversified rVV-plus-ALVAC approach described in the section entitled “CEA” under “Anti-idiotype Vaccines.” Active immunization can provide a polyclonal T-cell population specific for the tumor Ag that can be expanded and used in adoptive immunotherapy. By extrapolation from the experience with vaccines for infectious diseases, active immunization would be expected to have the greatest chance of therapeutic efficacy against minimal disease rather than against a rapidly growing, drug-resistant tumor. Preclinical investigations have demonstrated that eradicating established tumors will require the generation of high levels of tumor-specific immunity, levels that cannot be achieved by vaccination but rather must be achieved by infusion of competent T cells, i.e., adoptive T-cell therapy (103). Repeated vaccination will increase the number of immune effector cells, but eventually a plateau of responsiveness is reached, after which repeated immunizations do not appreciably change this level. Adoptive
transfer of T cells has resulted in one of every two host lymphocytes being of the infused cells' lineage (103). Adoptive immunotherapy may allow achievement of immunity at a level that may be effective against bulky disease.

Amplification of the Activation Status of the CTL Effector. To achieve effective responses, vaccination is expected to induce fully differentiated effector cells that can mount a quantitatively stronger response to tumor—higher levels of IFN-γ secretion and higher tumor lytic ability—than their precursors. The transition from a CD28+CD27+ to a CD28−CD27− state seems to correlate with an increase in perforin levels (104). Both effectors that are induced at priming and memory effectors share this phenotype. However, it is still not clear how to optimally induce CTLs to differentiate into effectors. For example, high-affinity melanoma-specific CTLs have been identified in vitro or in vivo (105, 106). Repeated in vitro stimulation with Ag has been shown to increase the ability of low-avidity cells to bind Ag dimer/tetramers; perhaps repeated vaccinations could have similar effects in the activation of high-avidity effector cells. The possibility of improving the ability of Ag stimulatory was recently proven by advances in the molecular modeling of TCR/foreign-peptide MHC complexes leading to Ag "repair" (107).

The differentiation program of effector cells (and that of memory CTLs) seems to be controlled by chromatin reorganization during cell division, and the outcome differs according to whether the division was induced by cytokines or by TCR. Stimulation by high amounts of Ag presented on large numbers of APCs is necessary but not sufficient for the induction of terminal effector differentiation. Fully differentiated effector cells that have been overstimulated cease proliferating and die by apoptosis upon restimulation with the Ag in various contexts. Only a few "memory effector" cells survive in the peripheral tissues. Alternatively, a weak initial TCR signal may promote the survival of effectors through the up-regulation of Bcl-2 and protect them from apoptosis at encounter with the Ag on tumor; however, in this situation the effectors would expand less and would not become fully differentiated effector cells.

The issue of how best to prompt the terminal differentiation of effector cells remains unresolved. According to one hypothesis, this can be achieved by preferential expansion of high-avidity CTLs that should divide, expand, and differentiate at the encounter with the tumor or the tumor Ag (105, 106). An approach to the first hypothesis is to use modified Ag that forms low-avidity TCR/foreign-peptide MHC-I complexes for priming and boosting; theoretically, the low-avidity ligands will allow selection of the high-avidity TCRs. An approach to the second hypothesis would be to use superagonists that form higher affinity TCR/foreign-peptide MHC-I complexes for vaccination. A potential problem with the first approach is that it leads to slow cell division (similar to homeostatic proliferation), and thus the clonal burst is small. A potential problem with the second approach is that the superagonists may overactivate certain response pathways but not others (e.g., IFN-γ secretion), resulting in massive CD95-mediated apoptosis and autoimmunity at various sites. If the effectors generated by each approach encounter the wild-type Ag on the tumor, and that wild-type Ag is a weak or partial agonist, then inactivation and death of the effector cells (tolerization) will spread, without the expected clinical results.

Nevertheless, these approaches hold great promise if they are selectively applied. For example, activation of high-avidity effectors by weak agonists should be useful for patients with no evidence of disease or a low leukocyte count. This approach may preferentially induce central memory cells. Activation of effectors by superagonists may be desirable in advanced disease with a widespread tumor burden, where immediate effectors are expected to be induced by vaccine. A combination of these two approaches may have synergistic effects in producing clinical responses. For example, vaccination with weak agonists after surgery, chemotherapy, or radiation should help replenish the pool of sensitized CTL precursors and increase the levels of antiapoptotic Bcl family members. This strategy should also offer protection from apoptosis because the CD95 ligand signaling will be stimulatory. Once a critical number of tumor Ag-specific CTL precursors has been induced, that number could be maintained by administering small amounts of cytokines such as IL-15, IFN-α, or IL-7. In this situation, use of superagonists would induce limited apoptosis of effectors because the up-regulated Bcl-2 and Bcl-XL could provide better protection than that observed in naive cells.

Development of selective and patient-tailored cellular therapies requires a novel approach to designing the immune-agonist and the mode of its delivery. The novel agonists should be able to induce mobilization and differentiation of CTLs to terminally differentiated effector cells. The need for such effectors has been demonstrated in persistent viral infections (104). These approaches require computer-directed modeling of the side chains of the tumor Ag, with fine-tuning of the agonistic effects achieved by controlled increases or decreases of the weak van der Waals forces through CH2 modification. Novel expression vectors are needed to deliver Ag directly to APCs to avoid cross-tolerization.

Coexistence of tumors with activated Ag-specific T cells in the presence of tumor-binding Ab indicates the induction of an immune response that is too weak, in numbers or in concentration, to mediate a therapeutic effect. In most patients classified as having no evidence of disease, the disease recurs after various intervals and cannot be controlled by existing long-lived peripheral memory and activated effectors. In viral infections, immunological memory is maintained by stimulation of the central memory T and B cells by other viruses over time. Moreover, ongoing studies of patients with no evidence of disease who are at high risk of relapse (48) or those with minimal residual disease (108) will provide the foundation for development of vaccines to extend patient survival or even for use in cancer prevention.

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