

Gene therapy strategies utilizing carcinoembryonic antigen as a tumor associated antigen for vaccination against solid malignancies

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Summary

Advanced solid malignancies represent a significant clinical problem with few effective treatment options. Carcinoembryonic antigen (CEA) is a well defined tumor-associated antigen on the surface of many solid malignancies that is currently used as a diagnostic and prognostic marker. Recent advances in tumor immunology, the understanding of antigen presentation, and gene transfer vector systems now provide gene therapy strategies in the form of a cancer vaccine which target CEA in an effort to induce a therapeutic immune response. This chapter provides a brief history of cancer vaccine development, discusses current strategies for generating or augmenting CEA-specific immunity, and focuses on ongoing CEA-based gene therapy approaches for the treatment of solid malignancies

I. Introduction

Advances in early detection, surgical technique and chemotherapy have improved treatment options for many solid malignancies such as colorectal, non-small cell lung, and breast cancer. Despite these gains however, the overall mortality rates for these patients have remained relatively stable over the past two decades and continue to account for 60% of annual cancer deaths (Landis 1998). Additionally, once past a surgically resectable stage I or stage II disease, effective radiotherapy and chemotherapy options are limited and survival rates decrease rapidly. Clearly, novel therapeutic approaches are needed for advanced solid malignancies.

Gene therapy in the form of a cancer vaccine may provide new treatment options for patients with these advanced stage cancers. An increasing number of human tumors are being shown to display specific tumor associated antigens (TAA) on their surfaces in combination with the major histocompatibility complex (MHC-I). These TAA have the capability of being recognized by cytotoxic T-lymphocytes (CTL) and therefore may function as targets for a tumor specific immune response. Melanoma derived antigenic peptides

from proteins such as MART-1, MAGE-1, MAGE-3, gp100, tyrosinase, and b-catenin have been detected by their ability to sensitize peptide-pulsed target cells to lysis by TIL-derived CTL lines (Kawakami 1994, Van der Bruggen 1991, Kawakami 1994, Cox 1994, Robbins 1996). Furthermore, several different TAAs including HER-2/neu, muc-1, PSA, and CEA have been identified in solid malignancies (Jerome, 1991, Peoples 1995, Ioannides 1993, Tsang 1995, Correale 1997). Although the *in vivo* significance of each of these antigens in T-cell immune response against cancer is yet to be defined, it is clear that CTL, which specifically recognize tumor antigens in the context of MHC-I, do exist *in vivo*.

Initial clinical work utilizing adoptive transfer of cytotoxic T-lymphocytes capable of specifically recognizing TAA led to tumor regression in select patients, demonstrating that a clinically relevant tumor regression can be mediated by TAA specific CTL (Rosenberg 1986). Moreover, recent work, performed in murine models and in patients with metastatic melanoma utilizing TAA epitopes now provide *in vivo* evidence that a cancer therapy approach based on relevant tumor associated antigens alone can be effective. Recent clinical studies using melanoma derived

antigens for vaccination have reported a 42% response rate in patients with advanced disease, closely correlating with the *in vitro* cytotoxic response to the TAA (Rosenberg 1998). These studies and others have, therefore, provided a great impetus for further development and design of TAA-based cancer vaccines (Mandelboim 1994, Feldkamp 1995, Meleif 1995).

Capitalizing on these recent advances in tumor immunology and antigen presentation by combining them with currently available gene transfer vector systems now provides gene therapy vaccination options which may generate or augment an *in vivo* TAA-specific CTL population. Such approaches have the potential to exploit the exquisite specificity of the immune system to target both primary lesions and metastatic colonies with lower toxicities than current treatment options. Furthermore, an immunomodulatory gene therapy approach would allow a relatively small population of transfected cells to induce a systemic immune response against cancer. This represents a significant advantage over classical gene replacement therapies which may require the transfer of gene constructs into every cell within a tumor, a feat currently beyond existing vector technologies. Gene therapy in the form of a cancer vaccine would also benefit from ease of administration, off-the-shelf availability, lack of a need for *ex vivo* manipulation, and would allow for the presentation of TAA epitopes away from the immunosuppressive effects of the local tumor site. Consequently, the majority of current cancer gene therapy protocols are now focusing on immunomodulatory approaches to develop a "cancer vaccine" (Sikora 1997).

II. Background

There is a long history of clinical trials attempting to immunize cancer patients with various cell preparations and tumor extracts. One early recorded attempt at cancer vaccination occurred in 1777 when Nooth, Surgeon to the Duke of Kent, inoculated himself repeatedly with cancer tissue. Ailbert, physician to King Louis XVIII, subsequently injected himself with breast cancer tissue in 1808. The recorded clinical outcome in both cases was no effect, ill or otherwise (Lyons 1987). In 1893, William Coley vaccinated cancer patients with a bacterial extract (Coley's toxin) to induce a general systemic immune response in hopes that the tumor would be attacked in a nonspecific manner (Coley 1893).

More recently, there has been a significant body of vaccination work performed by several investigators. Mastrangelo's group utilized autologous, enzyme dissociated, irradiated tumor cells combined with the adjuvant bacillus Calmette-Guerin (BCG) as a cancer vaccine following cytoxan treatment. After repeated dosing, positive delayed type hypersensitivity skin tests

(DTH) were seen against melanoma (Berd 1986, Berd 1990). Mitchell's group reported objective responses in 4 of 25 and 5 of 17 patients using a similar approach of an allogenic melanoma cell line lysate with the adjuvant DETOX™ (Mitchell 1988, Mitchell 1990).

Other groups have treated solid tumors in colon cancer patients by using enzyme dissociated, live, irradiated tumor cells combined with BCG. In low burden colon cancer patients this method gave delayed hypersensitivity responses against tumor cells and found a reduced relapse rate in some patients (Hoover 1985, O'Boyle 1992). Hollingshead reported that a partially purified "TAA" preparation generated by sonicating allogenic colon cancer cells and combining the extract with complete Freund's adjuvant similarly generated a DTH response (Hollingshead 1985). Although the results of these and many other studies did not provide a significant clinical benefit to the patient with a solid malignancy, they did discover that a cell mediated immune response is more effective in eliciting an anti-tumor effect than a humoral immune response. As a result, subsequent studies attempted to refine vaccine components in an effort to generate a cell mediated anti-tumor response against tumor associated antigens.

The majority of recent advances in this field have been generated by the discovery of interleukins and subsequent culture of T lymphocytes. Initial work by Yron *et al.* demonstrated that lymphocytes from murine spleens could be transformed into non-specific cytotoxic cells by incubation with high concentrations of IL-2. These lymphokine activated killer cells or LAK cells were shown to lyse weakly immunogenic tumor cells *in vitro* (Yron 1980, Rosenstein 1984). Preclinical experiments demonstrated that injection of LAK cells with IL-2 could reduce growth rate and prolong survival in murine models of metastatic lung and liver cancer (Mazumder 1984, Mule 1984, Mule 1985, Lafreniere 1985). Subsequent phase I and II human clinical trials were then conducted administering intravenous IL-2 and LAK cells with a 35% and 21% objective response rate noted in renal cell carcinoma and metastatic melanoma, respectively (Rosenberg 1991, Rosenberg 1992). Treatment with LAK cells and IL-2 also demonstrated activity against colorectal cancer in a small number of patients.

Efforts were then made to identify the specific cell population responsible for this anti-tumor effect, resulting in the isolation of tumor infiltrating lymphocytes (TIL) from both human and murine cancers (Galili 1979, Rosenberg 1986, Whiteside 1986, Spiess 1987). These T-cells were able to effectively treat tumor bearing animals (Rosenberg 1986), and could mediate tumor regressions in select patients with metastatic disease. A series of clinical trials performed at the NCI showed an objective response rate for TIL therapy of up

to 38% in patients with metastatic melanoma (Kawakami 1992, Kawakami 1994). These trials have successfully established that enhancement of the cell-mediated response of the human immune system can lead to cancer regression (Rosenberg 1995, Rosenberg 1997, Rosenberg 1988).

Tumor-specific cytotoxic T-lymphocytes (CTL) derived from TIL that could specifically recognize and respond to both autologous and allogeneic tumor cells in an MHC restricted manner have now been isolated *in vitro* (Rosenberg 1988, Rosenberg 1995, Rosenberg 1997). Recent progress in our knowledge of antigen processing/presentation and techniques for the isolation of peptides presented in an MHC-restricted fashion has led to the identification of tumor associated antigens (TAA) recognized by these T-lymphocytes (Topalian 1989, Darrow 1989, Wang 1995, Kawakami 1994, van der Bruggen 1994, Cox 1994, Robbins 1996). Several of the genes encoding for TAA's have been cloned, their class I MHC restricted epitopes described, and in some cases the functional specificity of T-cell receptor heterodimer recognition characterized (Van der Bruggen 1991, Rock 1993, Cole 1994, Cole 1995). These findings support the concept that the observed CTL-mediated tumor regression *in vivo* can be explained by the T-cell recognition of specific 9 or 10 amino acid peptides bound to MHC class I molecules presented on the surface of cancer cells. This represents a major step forward in cancer immunotherapy, and has provided the reagents to utilize specific tumor-associated antigens or peptides for vaccination therapy (Mandelboim 1994).

In order for T-cell based immunotherapy to be of benefit to the cancer patient, an appropriate TAA marker or markers must be selected as a target, and then this target must be presented to the patient's immune system in such a way as to produce a clinically relevant CTL response to the tumor. In the field of solid malignancies, several different TAA including muc-1, PSA, HER-2/neu, and CEA have been identified. Each of these antigens has advantages and disadvantages as vaccine targets for various cancer types. Prostate specific antigen (PSA) is a cysteine protease, whose expression is normally limited to prostate tissue. This organ specificity allows the use of PSA as both a diagnostic and prognostic marker for prostate cancer, and has led to a substantial increase in the early detection of this disease. In addition to its role in early detection, PSA is also being investigated as a possible target for prostate-specific cancer vaccines. A recent clinical trial vaccinated prostate cancer patients with dendritic cells pulsed with prostate specific membrane antigen and found a decrease in serum PSA levels, an enhanced cell-mediated immune response against PSA, and a partial regression of cancer in some patients (Salgaller 1998). This same group is continuing to explore the use of a cocktail of prostate antigens along

with GM-CSF in efforts to improve PSA-based vaccines.

HER-2/neu is a member of the epidermal growth factor receptor family that is overexpressed in 20-40% of intraductal carcinomas of the breast and 30% of ovarian cancers (Berchuck 1990, Kern 1990, Slamon 1987, Yonemura 1991) where it is associated with a poor prognosis (Slamon 1989). The protein is normally expressed during fetal development and is also found at low levels in epithelial cells of many normal tissues (Press 1990). Several approaches to HER-2/neu cancer vaccines are under investigation (Reviewed by Disis and Cheever 1997) and show promise in eliciting therapeutic antibodies against HER-2/neu positive tumors.

MUC1 is a large O-glycosylated mucin polypeptide expressed at high levels in many human adenocarcinomas. Cancer-associated MUC1 has an altered pattern of glycosylation which exposes a series of extracellular, antigenic 20 amino acid tandem repeats to the immune system (Gendler 1990, Lan 1990). Various methods are being investigated to develop a MUC1-based cancer vaccine including, recombinant MUC1 expressing vaccinia virus (Hareuveni 1990, Acres 1993), MUC1 cDNA injection (Graham 1996, Pecher and Finn 1996), and recombinant MUC1-derived peptide immunization (Ding 1993, Apostolopoulos 1996, Samuel 1998). A mannan (polymannose)/ MUC1 peptide fusion protein induced a strong CTL response which led to MUC1⁺ tumor regression in mice (Apostolopoulos 1994). However, when human patients were vaccinated with the mannosylated MUC1 fusion protein they displayed high levels of IgG1 antibodies with a low cell mediated response (Karanikas 1997). This discrepancy has been explained by antibodies to the Gala(1,3) Gal epitope which cross-react with MUC1 (Apostolopoulos 1998). Humans, unlike mice, express high levels of anti-Gal antibodies which may divert MUC1 vaccination to a humoral response. It may therefore be necessary to target antigen presenting cells against MUC1 *in vitro* to avoid this anti-Gal induced humoral response (Apostolopoulos 1998).

Finally, carcinoembryonic antigen (CEA) is an oncofetal protein which is overexpressed in 90% of gastrointestinal, 70% of non small-cell lung, and 50% of breast cancers (2,3,4). As a result of its presence in a large proportion of solid malignancies, CEA is a very attractive target for cancer vaccine therapy. It is one of the few TAAs in solid malignancies with which we have a significant amount of basic knowledge, in addition to some of the most mature preclinical and clinical vaccine experience. From these trials, we can derive some initial insight into the immune response to TAA vaccination, and as a result, they will serve as the focus for the remainder of this review.

III. CEA as a tumor-associated antigen target

CEA was first identified by Gold and colleagues in 1965 as a fetal antigen which becomes re-expressed in neoplastic cells (Gold 1965). CEA is a 180 kDa cell surface glycoprotein that plays a role in cellular adhesions, cell to cell interactions, and glandular differentiation (Bebchimon 1989, Pignatelli 1990). It is a member of a large family of glycoproteins that are expressed in fetal, normal adult, and malignant tissues (Von Kleist 1972). Several of these family members share antigen cross reactivity with CEA. Non-specific cross reacting antigen (NCA) located on normal neutrophilic leukocytes, normal fecal antigen (NFA-1), and bile antigen (BGP-1) are all weakly cross reactive with anti-CEA antibodies. More strongly cross reactive family members include normal fecal antigen 2 (NFA-2) and the non-specific cross reacting antigen 2 (NCA-2) found in meconium (Von Kleist 1979). In a healthy adult, CEA itself is expressed at very low levels in normal gastrointestinal crypts and in healing intestinal mucosa. Normally, CEA is only weakly antigenic with undetectable anti-CEA antibody levels in normal patient serum (Foon 1995, Schlom 1996). In contrast, the sera of some cancer patients contain CEA-immunoglobulin immune complexes in the thousands of ng/mL yet their tumor remains.

The goal of a T-cell based immunotherapy for solid malignancies is to increase the immunogenicity of this natural antigen until a clinically significant CTL response can be achieved against CEA expressing tumors. Many different approaches have been investigated in an effort to augment this T-cell based immune response to CEA including polynucleotide vaccinations, anti-idiotypic antibodies, peptide pulsed dendritic cells, and recombinant vaccinia virus infection. Each of these techniques shows promise as a potential treatment of solid malignancies.

A. Polynucleotide vaccinations

It has been proposed that direct DNA immunization might best mimic the circumstances of TAA overexpression by a tumor (Conry 1995). Vaccination by DNA immunization allows for persistent high-level protein expression *in vivo* and early results have been promising. Myofiber cells in the mouse were shown to express foreign genes that have been injected into muscle in the form of naked DNA without any cationic lipids, retroviruses or other special delivery systems (Wolff 1990). The duration of gene expression in skeletal muscle using a RSV promoter driving a luciferase reporter exceeded 19 months post injection, even though the foreign plasmid DNA appeared to remain episomal

(Wolff 1992). Naked plasmid DNA encoding influenza A nucleoprotein (NP) delivered to mice by IM injection produced influenza NP-specific antibodies and CTL response with protection from subsequent challenge with influenza A virus (Ulmer 1993). DNA-coated microprojectiles have also been used to vaccinate rodents and non-human primates with a variety of HIV-1 encoded antigens and both cellular and humoral immune responses resulted (Coney 1994).

More relevant to the treatment of solid malignancies, Conry's group has demonstrated lymphoblastic transformation and lymphokine release to human CEA using intramuscular injection of cDNA for human carcinoembryonic antigen into mice (Conry 1995, Conry 1995b). Furthermore, this naked DNA injection protected animals from tumor challenge with 2×10^5 syngeneic, CEA expressing tumor cells with no evidence of local toxicity or inflammation systemically or at the injection site. These results attest that DNA immunization holds great promise; however, immunization with DNA capable of integrating into the host genome raises significant safety concerns. The novel approach of mRNA immunization may avoid the possibility of integration and thereby overcome this safety concern. Preliminary studies using CEA have shown that mRNA immunization can generate CEA-specific antibody responses (Conry 1996). Further refinements in safety, expression promoters, methods of *in vivo* transfection, and the concurrent expression of cytokines and/or costimulatory molecules such as IL-2 and B7 will likely improve this direct polynucleotide injection approach to cancer vaccination.

B. Anti-idiotypic antibodies

Anti-idiotypic antibodies are another method being investigated as a means of establishing an anti-CEA immune response. Anti-CEA monoclonal antibodies have been used primarily for clinical diagnosis of colorectal cancer, either as a tumor marker in serum to monitor tumor recurrence, or as a means to localize CEA-bearing tumors and metastases in patients (Hardman 1992).

An additional application of antibody technology is the generation of anti-idiotypic antibodies that mimic CEA epitopes. Immunization with a given tumor-associated antigen (CEA) will generate antibodies against this antigen termed Ab1. The variable regions of Ab1 contain determinants known as idiotypes (Id), which are themselves immunogenic. Thus a series of anti-Id antibodies or Ab2 can be generated by injecting Ab1 into naive animals. Some of these Ab2 can effectively mimic the three dimensional structure of the CEA epitope identified by Ab1. Thus administration of Ab2 to cancer patients may generate an immune response to specific epitopes of CEA without generating non-specific cross

reacting responses to other family members.

An anti-Id antibody designated 3H1 mimics a biologically and antigenically distinct epitope of CEA, but not CEA family members found on normal tissues such as NCA (Bhattachary-Chatterjee 1990). 3H1 was capable of inducing CEA-specific antibodies in mice and rabbits, and a preclinical study has begun in cynomolgus monkeys (*Macaca fascicularis*) using aluminum hydroxide precipitated 3H1. Monkeys injected with 3H1 develop specific anti-anti-Id (Ab3) responses that were capable of inhibiting binding of 3H1 to Ab1. In addition, immune sera from monkeys contained Ab3 that bound CEA-positive carcinoma lines but not to CEA-negative cell lines. The induction of these anti-tumor antibodies in monkeys did not cause any apparent side-effects (Chakraborty 1995).

The monoclonal antibody 3H1 was then tested as a method for CEA vaccine therapy in 12 human patients with advanced colorectal cancer. Each of the patients received four intracutaneous injections of aluminum-hydroxide-precipitated 3H1. Nine patients demonstrated a CEA specific anti-anti-idiotypic response. Seven out of 12 patients demonstrated idiotypic-specific T-cell proliferation responses and 4 showed T cell proliferation to CEA (Foon 1995).

C. Peptide pulsed dendritic cells

Peptide pulsed dendritic cells comprise a recent approach to cancer vaccines which is receiving a great deal of attention. Dendritic cells (DC) are professional antigen-presenting cells who function to present antigen to naive T cells. In the past, DC have been shown to stimulate both a naive and memory T-cell response *in vitro* (Inaba 1990, Mahta 1994). Recent studies demonstrated that vaccination of mice with DC pulsed with TAA-derived peptides was highly effective in priming cytotoxic T-lymphocytes responses, and established both a protective and therapeutic anti-tumor immunity in treated animals (Huang 1994, Porgador 1996, Boczkowski 1996, Zitvogel 1996, Paglia 1996). Human studies have found that DC pulsed with TAA proteins can induce a CTL response *in vitro* (Macatonia 1991, Mahta 1994, Bakker 1995) and can produce a measurable cellular immune response in some B-cell lymphoma patients (Hsu 1996). In the field of solid tumors, Alters *et al* have reported that dendritic cells pulsed with the HLA-A2 restricted CEA-derived CAP-1 peptide can generate a CEA-specific CTL response as measured by a restricted T cell receptor repertoire in non-immunized pancreatic, colon, and breast cancer patients as well as in healthy volunteers (Alters 1998). Current studies are attempting to improve on these initial dendritic cell-based vaccines. In one such approach, dendritic cells treated with a proteasome

inhibitor or with antisense to TAP-2, transporter associated with antigen presentation, demonstrated an increased density of MHC I expression on their surface which led to a more effective vaccine (Wong 1998).

D. Recombinant Vaccinia vaccination

One of the best studied and currently popular methods of generating an anti-CEA T cell mediated immune response is the use of a recombinant vaccinia virus. This direct immunologic approach to CEA-bearing tumors was initially developed by the Laboratory of Tumor Immunology and Biology at the NCI using inoculation with a recombinant vaccinia virus (rV-CEA) that expresses the human CEA gene (Kaufman 1991). Vaccinia was chosen for this effort due to an intense inflammatory response generated at the site of infection which leads to both a humoral and cell mediated immune response (Bennick 1984, Moss 1987). Copresentation of a weakly immunogenic protein product at the site of vaccinia viral infection has been shown to elicit a strong "bystander" immune response against a variety of weak antigens (Lathe 1987, Hellstrom 1989).

Consequently, vaccinia is currently being investigated for use in immunizations against a wide range of infectious diseases as well as several types of cancer (Mackett 1987, Kierny 1984, Smith 1983, Langford 1986). A recombinant vaccinia expressing the HIV envelope protein has been administered to normal volunteers in phase I trials (Hu 1986, Cooney 1991) and constructs expressing tumor associated antigens have been tested in murine and non-human primate models (Estin 1988, Hu 1988, Bernards 1987, Hareuveni 1990, Hershey 1987, Kawa 1987).

A pre-clinical murine model for rV-CEA was initially established using a 2.4 kilobase cDNA segment coding for CEA (Oikawa 1987) inserted into the thymidine kinase gene of a WR (Kaufman 1991) and a NYC (Kantor 1992) strain of vaccinia virus. Cells infected with recombinant virus, rV-CEA, expressed CEA on their surface as detected by the anti-CEA monoclonal antibody COL-1. MC-38 murine adenocarcinoma was then transduced with the human CEA gene, causing CEA surface expression at levels comparable to those found on human colon cancer cell lines (Robbins 1991). Immune competent C57B / 6 mice were injected subcutaneously with 2×10^5 MC-38 cells or transduced MC-38 CEA cells. Seven days after tumor transplant, 10 animals with each tumor type were vaccinated with 1×10^7 plaque forming units (PFU) of either wild-type vaccinia or rV-CEA. Vaccinations were repeated twice at 14 day intervals. The animals inoculated with rV-CEA showed inhibition of growth of CEA positive tumor. In addition, mice which survived the initial MC-38 tumor challenge due to rV-CEA treatment did not allow growth of MC-38-CEA+tumor when re-

challenged (Kantor 1992).

The safety of rV-CEA was then tested in the rhesus monkey model (Kantor 1992b) because a successful immune response against CEA could result in an auto-immune colitis against endogenous CEA in gastrointestinal crypts. There is also a risk of auto-immune reaction against the cross reacting fecal antigens (NFA 1 and 2) and bile antigen (BGP-1) resulting in further intestinal and biliary inflammation. In addition, the expression of non-specific cross reactive antigen 1 (NCA) on normal neutrophils holds the possible side effect of leukopenia. Eight monkeys received up to 4 scarifications with either 1×10^8 or 5×10^8 PFU of rV-CEA and 4 monkeys received 5×10^5 PFU of control wild type vaccinia. All vaccinated monkeys developed typical local skin reactions, low grade fever, and lymphadenopathy after immunization. All rV-CEA vaccinated animals also exhibited strong anti-CEA responses, with no signs of auto-immune colitis and only minimal non-specific anti-NCA responses. Delayed type IV hypersensitivity responses were seen to intradermal injections of purified CEA in 7 or 8 recipients of rV-CEA, but none of the monkeys treated with wild-type vaccinia, indicating a specific cell mediated immune response. It should also be noted that cancer patients with high serum levels of anti-CEA immunoglobulin immune complexes do not show symptoms of immune complex deposition syndromes (Fuchs 1988).

IV. Initial Phase I clinical trials of rV-CEA

Based on this preclinical data, an initial phase I study was performed in patients with metastatic adenocarcinoma using an escalating dose administration of the rV-CEA vaccine (Tsang 1995). No Grade III or dose limiting toxicities were demonstrated in the study using doses as high as 1×10^8 PFU per vaccination. The only side effects to vaccination were a local, self-limited reaction at the injection site, lymphadenopathy, and low grade fever. A maximum tolerated dose, therefore, was not defined. Additionally, none of the potential problems of dose limiting leukopenia, auto-immune colitis, or toxic reactions to vaccinia itself were noted.

Although a therapeutic response was not realized in this trial, three important facts emerged. First, a series of HLA-A2 restricted peptides were identified which corresponded to the human major histocompatibility complex (MHC) class I restricted CTL epitopes within CEA. An immunodominant peptide identified in this series was the 9-amino acid (YLSGANLNL) CAP-1 peptide (Tsang 1995). Secondly, *in vitro* priming of post vaccination peripheral blood lymphocytes with the CAP-1 peptide in combination with IL-2 demonstrated MHC-restricted specific lytic activity against CEA expressing tumor cells in 5 of 5 patients tested (Tsang 1995)

(**Figure 1**). Thus immune recognition of CEA does occur in patients treated with the rV-CEA vaccine but at a sub-clinical level. Finally, presumably due to the high incidence of previous exposure to vaccinia within the population, it was found that an intense anti-vaccinia immune response followed the first vaccination. This inflammation produced neutralizing antibodies and inhibited replication of virus at the second and third administration, thereby limiting the ability of booster inoculations of vaccinia to expand the anti-CEA T cell population. Thus rV-CEA appears to be a self limiting but useful agent in inducing a CEA-specific anti-tumor immunity. Other methods may be needed, however, to boost this initial response to clinically significant levels.

Several different approaches are currently being investigated to augment this initial rV-CEA induced CTL population including the use of various cytokine and costimulatory reagents, avian pox virus vectors, and TAA-derived peptide booster inoculation. One of these "second generation" rV-CEA vaccination approaches combines a

Target cells	V24 effector T cells		
	CEA	HLA-A2	Specific Lysis
B cells	-	-	-
EBV-B A2	-	+	-
EBV-B A2/CEA	+	+	+
SW837	+	-	-
SW837 A2	+	+	+
SW403	+	+	+

Figure 1. MHC class I restricted specific lytic activity of post CEA vaccinated PBL. Patient post vaccination PBL samples (V24) stimulated *in vitro* with CAP-1 peptide displayed specific lytic activity by standard Cr⁵¹ release assay only against cells expressing both HLA-A2 and CEA. Targets included: autologous B cells (B cells); B cells transformed by EBV to express HLA-A2 (EBV-B A2); EBV transformed B cells expressing both HLA-A2 and CEA (EBV-B A2/CEA); the CEA positive, HLA-A2 negative colon cell line (SW837); the SW837 cell line transformed with HLA-A2 (SW837 A2); and the CEA positive, HLA-A2 positive colon cell line (SW403). Tsang *et al.* JNCI 87: 982, 1995.

recombinant vaccinia virus with various cytokine reagents. The cytokines IL-2, IFN-g, and TNF α are produced from the Th1 subset of CD4⁺ lymphocytes and normally function to induce a cell-mediated immune response. Although other cytokines were ineffective,

exogenous IL-2 when added in combination with a TAA-based pox virus vaccine, enhanced the immunogenicity of the tumor antigen and led to a decrease in pulmonary metastasis in animal models (Bronte 1995). GM-CSF is a potent cytokine which induces the differentiation of hematopoietic stem cells into dendritic cells and then promotes dendritic cell activation and differentiation at the local vaccination site. Studies adding GM-CSF to TAA-based cancer vaccines resulted in enhanced TAA immunogenicity (Dranoff 1993) and a recent clinical trial in renal cancer patients demonstrated a significant increase in DTH response when GM-CSF was added to vaccine formulation (Simons 1997). Recent studies have also focused on the heterodimeric cytokine IL-12, which also functions to shift a Th2 generated humoral immune response to the more effective Th1-based cell-mediated immune response. A vaccine composed of the mutated p53 protein combined with IL-12 led to the regression of sarcoma in one animal model (Noguchi 1995), and IL-12 combined with a recombinant vaccinia virus led to a decrease in metastases and a significant survival benefit in a murine model of adenocarcinoma (Rao 1996).

A related method of augmenting the rV-CEA induced immune response involves the introduction of costimulatory molecules into cancer vaccines. Specialized costimulatory molecules such as B7-1 and B7-2, which are expressed by antigen presenting cells, are thought to enhance activation and clonal expansion of T lymphocytes in a manner similar to cytokines. Moreover, it is widely held that T cell recognition of TAA in the absence of a costimulatory signal may lead to anergy and immunological tolerance. Transfection of tumor cells with B7-1 or 2 has increased the immunogenicity of some TAAs and has led to an anti-tumor effect in some animals (Townsend 1993, Guinan 1994, Baskar 1995).

An alternative method to boost the CEA-specific CTL population involves the use of an avian pox virus. Avian pox viruses are able to infect and express transgene in mammalian cells, but unlike vaccinia virus, avian pox viruses are not able to replicate in human cells. As a result, these vectors do not suffer from the dose limiting inflammation and neutralizing antibodies seen with vaccinia, and consequently, avipox vector can be given repeatedly. In addition, avian pox viruses can be safely administered to immunosuppressed patients, a current limitation of vaccinia use. Canary and fowl pox viruses have proven safe in extensive clinical trials as a possible rabies vaccine in both Europe and the United States (Taylor 1991, Taylor 1994, Cadoz 1992, Fries 1996), and a canary pox virus expressing the CEA protein (ALVAC-CEA) has been shown to induce an antibody response, a lymphoproliferative response, and a cytotoxic T lymphocyte response in murine models (Hodge 1997). Moreover, the combination of one rV-CEA vaccination

followed by two ALVAC-CEA booster injections resulted in a four-fold increase in CTL activity and prevented tumor formation in 5 of 8 animals (Hodge 1997).

A fourth method to augment the initial anti-CEA CTL population is booster vaccination with peptides such as CAP-1. Peptide-based vaccines offer a greater control over the ability to manipulate the immune response than many previous methods. Through the use of clearly defined immunogenic epitopes, peptide vaccines may elicit a CD4⁺ or CD8⁺ specific response as determined by the investigator. Peptide boosting also benefits from a relative ease of production, chemical stability, off the shelf availability, and lack of infectious or oncogenic potential (Aron and Horowitz, 1992).

The initial use of a MHC class I restricted vaccine was reported independently by two groups studying Lymphocytic Choriomeningitis virus and Sendai virus (Schulz 1991, Katz 1991). Work in cancer therapy quickly adopted this approach and animal data from the Laboratory of Tumor Immunology and Biology (LTIB) at the NCI, has shown that subcutaneous immunization of mice with 100 mg of short synthetic peptides (Ras5-17) demonstrated a specific T-cell immune response with no noticeable side effects (Peace 1991). The first use of a peptide vaccine in humans demonstrated that injection of a lipoprotein containing a HLA-A*0201-binding peptide from hepatitis B virus along with a pan HLA-DR binding protein could induce a strong CTL response (Vitiello 1995). Marchand *et al.* then showed that vaccination of melanoma patients with a MAGE-3 peptide could lead to a partial regression in some patients (Marchand 1995). Thus a TAA derived peptide vaccination may safely and under the proper circumstances, effectively boost a rV-CEA primed CTL population.

The mode of peptide based cancer vaccine administration, however, critically affects the ability to achieve a clinically relevant tumor-specific response. This can be explained in part by results showing that when a T cell encounters a TAA in the absence of B7-1 or B7-2 costimulation, the T cell becomes anergic or is tolerized to the antigen (Miller 1989, Ohashi 1991). This fact became apparent when vaccination with high doses of some TAA-derived peptides led to tolerance instead of protective immunity (Aichele 1995). Further study with an adenoviral induced tumor model found that vaccination with an adenoviral peptide, instead of eliciting a protective immunity, resulted in the induction of tolerance with a resulting increase in the outgrowth of the tumor (Toes 1996). These tolerizing vaccines clearly demonstrate that the local environment surrounding peptide vaccination is vital for the proper induction of an anti-tumor immune response.

Extensive experience in microbiology has shown that combining adjuvant reagents with peptide or protein

immunogens can prevent tolerance and lead to a productive immunization. The selection of the proper adjuvant for peptide immunization has a profound effect on antigen presenting cell activity at the local site of injection and therefore on the success of the vaccination attempt. Adjuvants may function by affecting the character and number of antigen presenting cells (APC) at the inoculation site, acting as a depot to prolong antigen/ APC exposure, or affecting the pathway by which proteins are processed (Allison 1994, Cole 1997). In the past, reagents such as BCG, Incomplete Freud's Adjuvant, or Detox™, have been shown to have an enhancing effect on both the humoral and cellular immune responses when used with vaccines (Ribi 1984). Detox™ has been used in several clinical trials with minimal side effects limited to flu-like symptoms and mild pain at the site of injection. A few patients who received Detox™ treatment have developed a granuloma at the site of injection but this spontaneously resolved and has not been a dose limiting side effect (Ribi, unpublished data). Therefore, administration of the CAP-1-peptide with the Detox adjuvant reagent may safely stimulate and significantly expand *in vivo* the number of CEA specific T-lymphocyte precursor cells present after rV-CEA vaccination. An enhanced CEA-bearing tumor T-cell population could then potentially lead to a direct therapeutic anti-tumor immune response. A gene therapy cancer vaccine approach was therefore initiated within the Department of Surgery Molecular Oncology Lab at the Medical University of South Carolina in collaboration with the NCI / LTIB for the treatment of patients with metastatic adenocarcinoma by administration of a rV-CEA vaccine followed by CAP-1 peptide boost in Detox™ PC adjuvant.

V. MUSC Phase I clinical trial: rV-CEA with CAP-1 peptide boost

rV-CEA (1.0 x 10⁸ PFU)

A phase I clinical trial was designed to investigate the effect of CAP-1 peptide boosting on the CEA-specific precursor T cell population established in patients initially vaccinated with rV-CEA. Because the pilot rV-CEA trial did not establish a maximum tolerated dose 1×10^8 pfu the highest dose tested in the original trial, was chosen as the initial vaccination dose. Additionally, intradermal administration rather than scarification was chosen based on recent data noting equivalent effectiveness for vaccine presentation (Galasso 1977, Wallack 1995). All patients received the rV-CEA vaccination on day 0 and again on week 4. This immunization was followed in four weeks by three rounds of CAP-1 peptide boosting on week 12, 16, and 20 (**Figure 2**). As a phase I trial, the study was designed for 12 patients in four groups of three peptide escalations. If grade III toxicity were noted at any peptide dose level the cohort would be doubled. The dose of CAP-1 peptide to be administered was 300 mg/mL for the first three patients and was then escalated to 6000 mg/mL in the final group. At 4 weeks post treatment, patients will be evaluated for complete response (CR), partial response (PR), stabilization of disease (SD), or progression of disease (PD). Follow up is weekly until 28 days after the final dose, and then monthly until disease progression or until initiation of any new form of therapy.

The patient population enrolled on study was defined by diagnosis of a histologically confirmed, CEA⁺ adenocarcinoma of the gastrointestinal tract, breast, or lung with expected survival of 6-12 months with no concomitant therapy. Due to the CAP-1 MHC restriction, all patients must further demonstrate HLA-A2 expression by tissue typing. Patients were also required to have a Zubrod performance score of 0-1 with serum CEA levels of >10 ng/mL, and normal immunological testing by DTH and CD4/CD8 ratio.

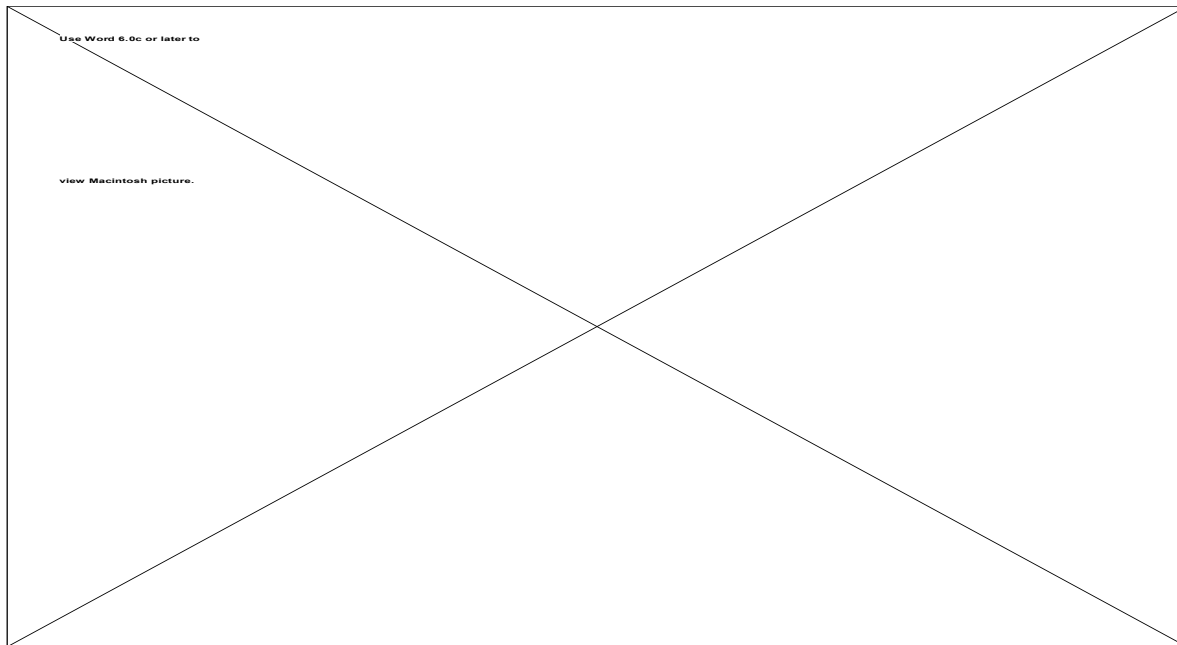


Figure 2. Treatment Schema. Patients receive 1×10^8 pfu of rV-CEA by intradermal injection on day 0 and again on week 4. This vaccination is followed by CAP-1 peptide boosting on week 12, week 16, and week 20. Patients are followed for signs of response to treatment with immunological responses determined before rV-CEA vaccination, before CAP-1 peptide boosting, and after CAP-1 peptide boosting is complete.

At time of publication, 10 patients have been enrolled in this trial. Although the data is insufficient to draw conclusions as therapy is ongoing, the patient results to date are presented in **Table 1**.

Off Study 3

* 5 patients show clinically stable disease with 2 patients showing progression of disease.

Table 1. Patient profile

<u>Patient Characteristics</u>	<u>Number</u>
Age (average)	52.8 yrs
Sex	
Male	3
Female	7
Primary Malignancy	
Colorectal	6
Lung	2
Gallbladder	1
Unknown	1
Prior Treatment	
Chemotherapy	5
None	5
Current Status	
Ongoing	7*

Evaluation of the immune response to a TAA-based cancer vaccine is currently a major hurdle in clinical cancer vaccine trials. Although it is clear that CEA-specific T cells are present after rV-CEA immunization, the clinical response in patients is unpredictable. Physical examination and radiological monitoring are unequivocal measures of response. Short of this however, a meaningful measure of a vaccine's effect on a patients T cell population is also instructive. Due to their exceedingly small numbers, it is rarely possible to measure TAA-specific CTL precursor populations in patient peripheral blood samples (Coulie 1992, Marrocchi 1994, Herr 1994). The assays presently employed in attempts to monitor the immune response to cancer vaccination include delayed type hypersensitivity testing, measurement of T cell precursor frequency by thymidine incorporation and cytokine release, target-specific lysis by chromium release assay, and T cell receptor analysis by gene scan and competitive PCR. Unfortunately, none of these assays give an accurate picture of the T cell response to treatment in and of themselves.

Intradermal injection of irradiated tumor cells into a patient before and after treatment elicits a delayed type hypersensitivity response, and this DTH is the *in vivo*

assay most commonly used in clinical trials to follow T cell response to vaccination. Although a DTH assay is technically simple to perform and is generally present in patients displaying a measurable clinical response to treatment, the assay is not predictive of clinical response (Berd 1990).

Several *in vitro* assays are also used to evaluate T cell response to vaccination including cell proliferation, cytokine production, and chromium release assays. These methods measure CTL precursor frequency by culturing TAA pulsed or TAA expressing target cells with patient derived T cells. Limiting dilution techniques allow all of these methods to quantitate the precursor frequency in patient samples. The use of [³H]-thymidine incorporation provides a direct measure of precursor cell proliferation in response to TAA stimulation (Wucherpfennig 1995). The release of cytokines such as IL-2, IFN- γ , and TNF α from patient T cells grown in mixed culture is also used to measure the T cell response to TAA immunization. The Cr⁵¹ release assay is the most common *in vitro* assay used to monitor T cell response to cancer vaccines. However, during a recent clinical trial, patients who underwent complete remission of melanoma as a result of MAGE-3 peptide vaccination did not demonstrate any MAGE-3-specific CTL activity as detected by the chromium release assay (Marchand 1995). The chromium release assay also failed to detect

TAA-specific CTL activity in a trial involving a gp100 peptide which was modified to more tightly bind the MHC complex even though this peptide vaccination demonstrated a 41% clinical response rate in patients (Parkhurst 1996).

An alternative measure of T cell response to a TAA-based vaccine is analysis of T cell receptor subtype expression. T cells recognize MHC-restricted antigens through a heterodimeric T cell receptor (TCR) composed of α and β chains. Somatic recombination between variable (V), joining (J), and diversity (D) genes along with insertion of random N-nucleotides, generates a wide diversity of TCR subtypes in naive T cells. As a result, peripheral blood mononuclear cell samples from non-immunized patients display a roughly equivalent abundance of TCR variable β -chain subtypes (TCR-V β 1 through TCR-V β 24). In contrast, if a TAA-based vaccine induces a clonal expansion of T cells recognizing the antigen then a subsequent alteration in TCR subtypes expression patterns should result. In fact, TCR screening studies have demonstrated that CTL effector populations can display an oligoclonal expression pattern after peptide immunization and *in vitro* stimulation (**Figure 3**). Loftus *et al.* have shown that TCR-V β 14, along with V β 4 and V β 3 are sharply increased in peripheral blood lymphocytes (PBL)

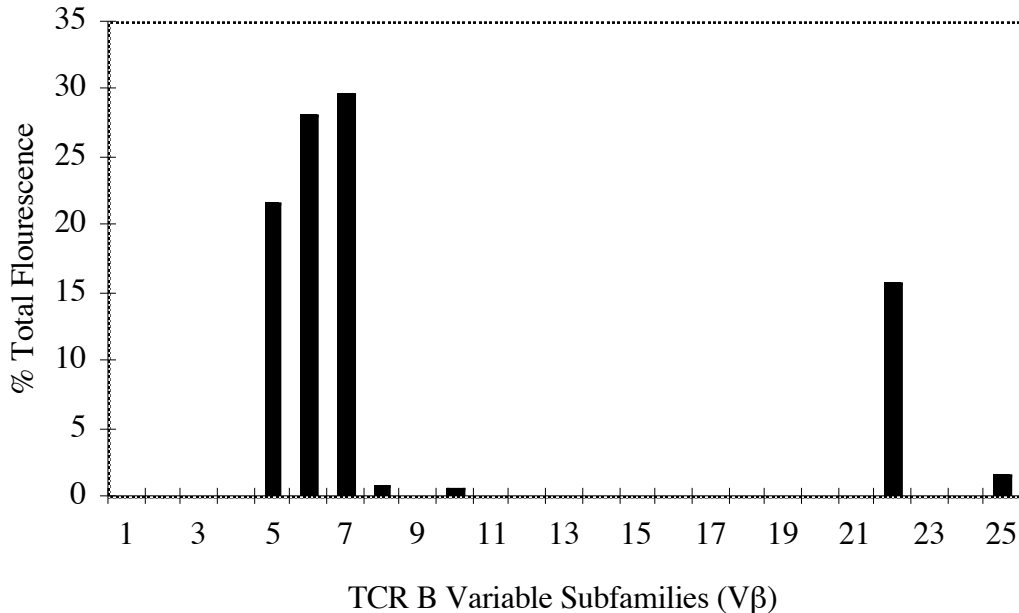


Figure 3. Post rV-CEA vaccinated PBL, stimulated *in vitro* with CAP-1 peptide display an oligoclonal expansion of T cell receptor V β family subtypes. Total cellular RNA was isolated from 5×10^6 V24 T cells. First-strand cDNA was then synthesized from 1 mg of total RNA and amplified with 25 V β oligonucleotides and FITC-labeled C β oligonucleotide. Labeled PCR products were loaded on a 6% acrylamide sequencing gel and the samples were then run on an ABI 373 sequencer for size and fluorescence intensity determination. The relative percentages of each V β subfamily are represented as histograms.

from peptide stimulated patients (Loftus 1996). Several groups have corroborated these findings and other studies have shown that Vb3 and Vb4 are increased in MART-1 peptide stimulated CTL (Cole 1994, Sensi 1995). These studies have clearly shown TCR changes post vaccination, however, a predictable patient to patient trend in subfamily response has not been observed (Cole 1997). RT-PCR is currently used to identify TCR family subtypes in these analyses, but due to different family-specific annealing temperatures, RT-PCR cannot be used to accurately quantitate various TCR expression levels. The advent of competitive PCR (cPCR) may overcome this difficulty and allow quantitation of specific TCR-Vb chain subtypes within CTL samples derived from PBL and TIL (Uhrberg 1996).

In the MUSC phase I clinical trial using rV-CEA vaccination with CAP-1 peptide boosting, patients will be evaluated by several different methods to determine both humoral and cell-mediated responses to treatment. Labs for *in vitro* testing will be drawn on week 0 before vaccination, on week 12 before CAP-1 peptide boosting, and on week 24 after peptide boosting is completed. Patient sample testing will be divided between the Laboratory of Tumor Immunology and Biology at the NCI and our laboratory at the Medical University of South Carolina (MUSC). The LTIB will evaluate humoral response to vaccination by standard ELISA assay for pre and post treatment levels of CEA, normal cross reactive antigen (NCA), anti-vaccinia, anti-CEA, and anti-NCA antibodies. The level of CEA-anti-CEA immune complexes, CD3, CD4, and CD8 subsets will also be measured. The LTIB will also study T cell precursor frequency in pre and post treatment samples by using limiting dilution assays for [³H]-thymidine incorporation and microtiter ELISA cytokine release as previously described (Abrams 1995). MUSC will monitor T cell receptor family subtype alterations by a combination of gene scanning and competitive PCR techniques to follow any T cell-mediated response to treatment. The optimal dose of peptide will be determined as the lowest level which elicits the highest proliferation or cytotoxic response in all members of a group.

VI. Conclusions

Advances in tumor immunology are now combining with gene therapy techniques to provide promising new therapeutic options for the treatment of patients with solid tumors. There are currently several TAA involved in solid malignancy, including PSA, HER-2/neu, MUC1, and CEA which hold potential for future vaccine development. Of these, CEA has received perhaps the most attention as a target antigen for cancer vaccines by numerous methodologies. Previous studies with rV-CEA

have proven safe with no evidence of autoimmune or other severe toxicity, and although a clinically relevant response has not yet been achieved, the clear demonstration of a CEA-specific CTL population in vaccinated patients represents a scientific success. Clinical trials using many vaccine strategies are now in progress in an effort to expand this CEA-specific CTL population to clinically beneficial levels. A CAP-1 boosting approach to augment the rV-CEA generated anti-CEA CTL population has been initiated, but the effectiveness of the method has yet to be determined. It is clear however, that cancer vaccine-based gene therapy holds tremendous promise and may one day provide an effective treatment for patients with solid malignancy.

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