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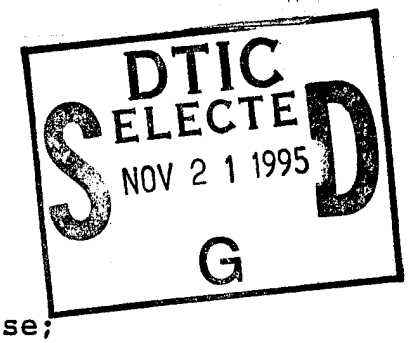

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The underlying reason for failure to cure patients diagnosed with breast cancer is the presence of micrometastases in approximately one third of the breast cancer patients. The stimulation of anti-tumor immune responses represents one of the most effective ways to treat low tumor burdens that are microscopically or clinically occult. The objective of our proposal is to determine whether a poliovirus replicon vaccine strategy engineered to express human CEA and, in the future, the HER2/neu oncogene, can induce systemic immunity and eradication of micrometastases.

During the first year of our project, we have constructed poliovirus replicons which express CEA. Mice given these replicons generate anti-CEA antibodies. We have constructed replicons which contain the extracellular domain of the HER2/neu oncogene and will be testing the immunogenicity in mice. The significance of these studies is that we have now characterized the appropriate replicons that will be used to establish route of administration for generating an immune response against CEA or HER2/neu prior to tumor challenge. The results of these studies will provide essential preclinical observations that will be relevant to the starting of human breast cancer trials targeted against cells that express CEA or HER2/neu.

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Craig Moore 9/13/95

PI - Signature Date

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INTRODUCTION

The frequency of micro-metastatic cancer deposits has correlated with a number of factors at the time of primary therapy of breast cancer including regional lymph node metastasis, size of primary tumor, tumor differentiation and certain molecular aspects of individual tumors including aneuploidy, DNA synthesis (S phase) and presence or absence of amplified gene expression for c-myc, p53, or HER2/neu. The use of systemic hormonal therapy, chemotherapy or combined adjuvant therapy directed at these micro-metastases has produced a reduction in metastatic relapse rate and improved survival. These effects are modest, and the majority of patients with micro-metastasis still have disease recurrence and ultimately die of metastatic breast cancer. **Thus, it is clear that additional strategies to eradicate micro-metastasis at the time of initial diagnosis is a major priority in breast cancer therapy research.** It should also be noted that our inability to detect occult micro-metastases means that patients with and without micro-metastases will be treated. The strategy should have low toxicity, ease of administration and cost-effectiveness (low cost).

Multiple animal model studies (19,20) have demonstrated that immune response to tumor associated antigens can have a dramatic antitumor effects, but such treatments strategies rapidly lose efficacy as progressive tumor growth occurs (progressive time after tumor implantation or metastasis). Thus, the induction of an immune response to tumor associated antigens in humans is likely to have limited success in patients with obvious metastases and its optimal application would be at the time of occult micro-metastasis as an adjunct to primary therapy. Active specific immunotherapy to enhance host immune response to tumor associated antigens has been called "vaccine" therapy although this application does not fit the strict (narrow) definition that entails prevention of disease rather than therapy of an existing disease. It is the purpose of this project to define a novel strategy to enhance antitumor response to tumor-associated antigens, leading to the development of therapeutic vaccines.

A variety of studies have demonstrated that immunization with tumor preparations can produce antitumor immune responses and efficacy in animal models (38,14,17). Similar studies in man have shown antitumor effects in patients with melanoma (5,33) and other tumors (29). The ability to isolate and clone putative tumor antigens provides the opportunity to utilize more defined reagents and to allow analysis of specific immune responses in guiding the design of active immunotherapy trials. A variety of potential targets include CEA, HER2/neu, MUC-1, MAGE 1, mutated RAS, mutated p53, etc. (20). A number of genetically engineered cancer vaccines utilizing cloned tumor associated antigens in vaccinia virus constructs or with adjuvants are undergoing clinical trials (7). We believe that this approach represents a fertile and novel new technology, and that we are just beginning to identify potential tumor associated antigens (and their genes) which will be applicable to novel strategies for enhancement of anti-tumor immune responses in animal models and man.

Poliovirus Replicons to Express Foreign Genes

The proposed experiments are based on the use of poliovirus as an expression vector for proteins to deliver antigens to immunoreactive sites of the immune system. Poliovirus is an RNA virus (no DNA intermediates) and is well-suited for these studies. The natural transmission of poliovirus is via an oral-fecal route (22). Once the virus is ingested, primary multiplication occurs in the oropharyngeal mucosa with subsequent spread into the tonsils, intestinal mucosa, Peyer's patches, deep cervical and mesenteric lymph nodes. Infection with attenuated strains of poliovirus results in viral replication and generation of systemic and mucosal immune responses, but, in contrast to the wild type virus, does not result in the CNS involvement associated with the wild type poliovirus infection.

Previous studies using the Sabin attenuated poliovirus strains have clearly established that both the systemic and mucosal immune systems are stimulated by administration of the oral vaccine, and that the immunity is long-lasting (35,44). Administration of poliovirus to mice has produced T cell immune responses including T helper cell proliferation, lymphokine release and cytolytic T cell responses (25,26,27). Antibody responses occur systemically (IgG and IgM) and at the mucosal surfaces, i.e., IgA (35). Normal adults have T cell lymphoblastic transformation to poliovirus (16,46). Follow-up studies of individuals immunized with the Sabin attenuated strains have demonstrated that circulating antibodies to poliovirus are maintained for at least ten years (45). Importantly, a comprehensive study of 175 children booster immunized with oral vaccine revealed that a specific IgG response was observed in the majority of the subjects who had not been vaccinated for at least 5 years (4). Primary immunization with oral poliovirus vaccines also appears to successfully prime for subsequent booster immunization, whether administered by oral or parenteral route (36). These studies support the contention that poliovirus has the ability to establish long-lasting immune responses involving both cellular and humoral immunity.

The availability of an infectious poliovirus cDNA has provided the opportunity to utilize molecular genetics to investigate various aspects of viral replication. Previous studies had demonstrated that deletion of the majority of the P1 coding region results in an RNA genome with the capacity to replicate when transfected into cells, provided the translational reading frame is maintained between the deleted regions (8,23,40). My laboratory has extended these studies and demonstrated that foreign genes can be inserted into the poliovirus RNA genome, resulting in the generation of replicons which replicate and express the desired foreign protein when transfected into cells (1, 2,40,41).

BODY OF THE PROPOSAL

The Specific Aims of the proposal have not changed since year 1 and are as follows:

1 To construct poliovirus replicons which express native and truncated CEA proteins (including secreted and non-secreted molecules (months 1-18).

2. To characterize and optimize the immune response to CEA elicited by both oral and parenteral administration of such vaccines in mice (months 4-24).

3. To test the ability of such poliovirus - replicon CEA vaccines to generate antitumor effects as measured by resistance to tumor challenge in a syngeneic murine CEA expressing breast cancer model (months 12-36).

4. To test the therapeutic effects of such vaccines in the eradication of breast cancer micro-metastasis in a syngeneic, spontaneously metastasizing CEA positive breast cancer model (months 18-48).

During the first 12 months, we have made considerable progress towards completion of Specific Aim 1. The majority of the results were presented in the manuscript :

Ansardi, D.C., Z. Moldoveanu, D.C. Porter, D. E. Walker, R. M. Conry, A.F. LoBuglio, S. McPherson and C.D. Morrow. 1994. Characterization of poliovirus replicons encoding carcinoembryonic antigen. Cancer Research. 54: 6359-6363 (reprints provided).

The manuscript describes the construction and characterization of poliovirus replicons which express the gene for carcinoembryonic antigen (CEA) (2). We demonstrated that transfection of this replicon into cells resulted in the replication of the RNA and expression of CEA. **Immunization of mice with these replicons resulted in the generation of an antibody response against CEA.** This result demonstrated the feasibility of using the poliovirus replicons as a means to induced immunity to a tumor associated antigen such as CEA.

In a second series of experiments, we have constructed replicons which contain a truncated version of the CEA gene that encodes an intact signal sequence without the membrane anchor region. We have demonstrated that the RNA genome containing this version of the CEA gene is replication competent and the CEA expressed from this replicon is glycosylated. We have derived stocks of the encapsidated replicon which encodes the CEA protein. Experiments are ongoing to determine if the glycosylated CEA is secreted from cells.

In the next period, we intend to immunize animals with the encapsidated replicons encoding the non-glycosylated and glycosylated versions of CEA. We have found that intramuscular immunization of animals results in a serum antibody response. Administration of the replicons by intranasal, oral or intrarectal routes results in antibodies in the secretions. We are using several doses of the replicons to optimize the conditions for generation of an immune response.

CONCLUSION

Expression of CEA using poliovirus replicons and analysis of immunogenicity.

The proposed studies for CEA are progressing on schedule. As pointed out in the Specific Aims, we are now establishing the parameters for immunization of mice by parenteral (intramuscular injection) and oral (or intranasal) routes.

Extension of current studies to expression of HER2/neu oncogene in poliovirus replicons (submitted to NIH as a supplement to Army grant DAMD 17-194-J-4403).

The success of the expression of CEA using replicons has prompted the extension of this system to other genes relevant to breast cancer. In particular, our studies have focused on the HER2/neu oncogene. The HER2/neu oncogene (also named erb-B2) has been demonstrated to be amplified in human breast tumors (34,47).

The amplification of the gene correlates with the expression of 185 a kDa transmembrane glycoprotein (10). Previous studies have demonstrated that the 185 kDa HER2/neu protein has a restrictive tissue distribution in fetal and normal adult tissues (34). In neoplasms, the 185 kDa HER2/neu protein was expressed in 46% of primary breast cancers, 28% of ovarian tumors and 30% of colon rectal malignancies. In metastatic breast tumors the 185 kDa HER2/neu oncogene was present homogeneously among multiple tissue lesions and 80% of the time the expression of this protein in the primary lesion correlated with that of the deriving metastases. Based on these studies, this gene product has been found to be of importance in the prognosis and potentially therapy of these malignancies.

The over-expression of the 185 kDa glycoprotein in transformed cells coupled with the restrictive expression of this protein in normal tissues has prompted the development of vaccine strategies against this protein (34). Previous studies have described the construction and characterization of recombinant vaccinia viruses which express the 185 kDa HER2/neu gene product (6). Immunization of mice with the recombinant vaccinia virus expressing this 185 kDa protein protected the mice from subsequent tumor challenge with cells expressing the rat neu oncogene (which is homologous to the HER2/neu oncogene). Further studies have demonstrated that immunization of animals with the extracellular domain of the 185 kDa HER2 gene product results in the production of both a humoral and cell mediated immune response against this protein (15).

Although the recombinant vaccinia strategy has been successful in mice, the application of this strategy to humans has several drawbacks, including the fact that the virus replication takes place in the skin and can be prevented by prior immunity. In addition, immunization with recombinant vaccinia encoding an oncogene has some biosafety concerns. Thus, alternative vaccine strategies utilizing putative tumor antigens are of general interest. **In particular, it would be important if these strategies could be incorporated into a vector which utilizes RNA rather than DNA as the replicating nucleic acid.**

In preliminary studies, we have constructed a poliovirus replicon which contains the complete gene for HER2/neu as well as a truncated gene which encodes the extracellular domain (ECD). Following linearization of the cDNA templates with *Sal* 1, we utilize *in vitro* transcription using T7 RNA polymerase for the production of RNA that is transfected into cells. We have utilized metabolic labeling followed by immunoprecipitation with antibodies to either the poliovirus polymerase (3CD) or monoclonal antibodies specific for HER2/neu. Previous studies from my laboratory have demonstrated that the expression of the 3CD protein correlates with the capacity of the replicons to undergo self-replication. Both of the replicons containing full length HER2/neu oncogene as well as the ECD of HER2/neu were replication competent when transfected into cells as shown by the production of the poliovirus 3CD protein. The replicon also expressed a protein that was immunoprecipitated with antibodies specific for the extracellular domain of HER2/neu. Expression of the HER2/neu gene product from the replicons containing the full length HER2/neu oncogene resulted in the expression of a protein that migrated at a molecular mass of approximately 185 kDa. The migration of this protein is consistent with that for the full length HER2/neu oncogene. The replicons which contained the extracellular domain of the HER2/neu oncogene also expressed a protein with a molecular mass slightly less than that for the complete HER2/neu oncogene (120-150 kDa).

Additional experiments proposed to incorporate the replicons which express the ECD of HER2/neu using poliovirus replicons.

We propose to extend our studies on the replicons which express CEA to those which also express HER2/neu. The proposed studies are warranted because of the relevance of HER2/neu to breast cancer. In addition, we have preliminary data that the expression of HER2/neu by a replicon is feasible and thus, should be possible to extend this work into animals in the same protocols as that for CEA. We have applied for a supplement to the current grant through the Public Health Service Office on Women's Health.

The following Aims are proposed for the HER2/neu replicons (similar to that for replicons expressing CEA).

Specific Aim 1. To characterize the expression of HER2 extracellular domain gene product.

Encapsidation of the replicon expressing ECD of HER2/neu.

The experiments in this section are designed to encapsidate the replicon containing the extracellular domain of the HER2/neu gene. Although we will attempt to encapsidate a replicon containing the complete HER2/neu gene (3.9kb), we anticipate that this replicon will not be encapsidated because the overall size of this replicon is approximately 1.5 kb larger than the complete poliovirus genome. Since the size of the replicon containing the extracellular domain of the HER2/neu oncogene is approximately 500 base pairs less than the complete poliovirus genome, based on our previous studies with over 20 foreign genes we anticipate no problems with encapsidation. For these studies, we will utilize standard methodologies that have been published previously in Porter et al., 1995.

Characterization of the HER2/neu ECD expressed from an encapsidated replicon.

Once we have established stocks of the encapsidated replicon containing the ECD for HER2/neu, we will perform experiments to characterize the expressed HER2/neu protein (see Appendix 3) Since the HER2/neu ECD does not encode a membrane anchor region, we would expect the HER2/neu ECD to be released from cells if it undergoes correct post translation modification. In the first series of experiments, we will perform a detailed pulse chase analysis to determine if the HER2/neu ECD is released into the medium of infected cells. Although we anticipate the secretion of the antigen would be expressed to favor antibody response, cell mediated immunity, particularly cytolytic T cell response, is probably pivotal to development of anti-tumor immunity. Cytolytic T cells typically recognize peptide fragments of antigen synthesized intracellularly and expressed in the center of MHC class I molecules without regard to glycosylation. Thus, intracellular synthesis of HER2/neu product directed by polio replicons would be expected to elicit cytolytic T cell responses to HER2/neu without regard to secretion or glycosylation of the protein. Previous studies have described generation of human T cells specific for peptides of the HER2/neu ECD (12).

Specific Aim 2. To optimize the immune response to encapsidated replicons encoding the HER2/neu ECD.

The establishment for an optimal dose of the poliovirus replicon expressing the HER2/neu ECD for use as an oral and parental vaccine is complicated. Our approach here will be to utilize similar experimental procedures similar to those proposed in the original funded grant application for CEA. Since we have already demonstrated that administration of the replicons alone expressing CEA results in generation of an immune response to CEA, we will establish a dose response curve for the replicons expressing HER2/neu ECD. These effects may be different for an oral as compared to a parenteral (intramuscular) dose so we will study both routes of administration.

Design of oral poliovirus replicon/HER2/neu vaccine dose finding study.

Group #	Infectious units of HER2/neu	
1)	10^5	(oral)
2)	10^5	(parenteral)
3)	10^6	(oral)
4)	10^6	(parenteral)
5)	10^7	(oral)
6)	10^7	(parenteral)

Each group will contain 10 mice. Five mice from each group will be sacrificed 3 weeks post administration for study of immune response. The remaining 5 mice will be given a second dose and will be sacrificed 3 weeks later (post-booster) for study of immune response. We will screen for immune response using serum antibody response to HER2/neu and splenocyte T cell proliferation to HER2/neu versus control proteins.

These broad dose ranging studies should give us an estimate of the best dose for each route of administration to carry forward to a comprehensive evaluation of HER2/neu immune response. Groups of 20 mice will be vaccinated orally or IM using the optimized replicon dose with 3 vaccinations given 3 weeks apart (days 0, 21 and 42). Fifteen mice in each group will receive replicons encoding HER2/neu while the other 5 receive replicons encoding HIV-1 Gag protein as negative controls. Five HER2/neu immunized mice will be sacrificed 3 weeks after the initial vaccination and 3 weeks after each booster immunization to obtain sera and spleen for evaluation of the HER2/neu specific immune response. The 5 control mice will be sacrificed 3 weeks after the second booster immunization. The immune analysis will include splenic T cell proliferation and lymphokine release (Il-2 and IL-4) to HER2/neu, inactivated poliovirus and control proteins; cytolytic T cells assays to HER2/neu positive and negative 4T1 tumor cells and antibodies to HER2/neu.

If the oral route of initial immunization and oral booster give poor systemic immunity, we will also evaluate oral initial immunization followed by intramuscular booster injections.

Finally, we ultimately want to move this vaccine strategy into a therapy model which will be described subsequently but which has a therapy window of 4-8 weeks between primary tumor resection and end stage metastases so that rapidity of immune response induction is an important issue. We will therefore examine an alternate schedule of administration using weekly oral or intraperitoneal administration of the optimal dose of

vaccine with comprehensive analysis of immune response to HER2/neu at 3 and 6 weeks as described above.

These studies will provide an optimal dose for oral and intramuscular administration as well as two schedules of administration to move into *in vivo* antitumor efficacy studies (Specific Aim #3).

Specific Aim 3. To test the ability of a poliovirus replicon HER2/neu vaccine to elicit protection against tumor challenge.

We plan on having *in vivo* antitumor efficacy data as a complement to the immune response analysis described in the prior section. Groups of 12 mice will receive the optimal dose of poliovirus replicon HER2/neu or poliovirus replicon HIV-1 Gag according to four schedules:

(1) orally every 3 weeks x 3
(2) orally weekly x 6
(3) intramuscular every 3 weeks x 3
(4) intramuscular weekly x 6

Six animals in each group will be tumor challenged on day 21 and six animals on day 42. Tumor challenge will utilize 2 x 10^5 HER2/neu transfected 4T1 cells on one side of the animal and an equal number of untransfected 4T1 cells on the contralateral side. We have selected a model originally developed by Gloria Heppner's group at Michigan Cancer Foundation from a spontaneously arising breast adenocarcinoma in a BALB/c mouse (11). Cell lines developed from this tumor have been used for a variety of studies relevant to tumor heterogeneity (30), drug sensitivity (21), local tumor growth (31), and metastasis (3,32) by Miller and Heppner. Dr. Miller has provided us with the 4T07 and 4T1 sublines derived from the 410.4 parent line along with advice on their *in vitro* and *in vivo* handling. Local tumor growth will be measured serially to determine the degree of protection against tumor challenge induced by the HER2/neu specific immune response. The growth of HER2/neu expressing tumors will be compared in mice immunized with HER2/neu versus HIV-1 Gag encoding replicons (40,41) as well as comparison with the contralateral HER2/neu negative tumor. This design will also provide information on the rapidity of antitumor effects *in vivo* by comparing the 3-week and 6-week challenged mice.

The 4T1 tumor cell line provides a very rigorous model for the evaluation of immunoprecipitation in that it is relatively non-immunogenic. However, it should be noted that immunogenicity relates to induction of an immune response. In these studies, induction of immune response has been carried out by a vaccine to HER2/neu. Thus, the 4T1- HER2/neu cells need to serve as a target, i.e., display of MHC-peptide (antigenicity rather than immunogenicity). It is our expectation that the MHC display on 4T1 cells will be sufficient to serve as targets for T helper and cytolytic T cells.

Specific Aim 4. To test the therapeutic effect of such vaccines in the eradication of breast cancer micro-metastases.

A plasmid encoding human HER2/neu and luciferase has been constructed in our laboratory and used to transduce the 4T1 line to yield 4T1- HER2/neu-Luc. The 4T1 line has also been transduced with a luciferase encoding plasmid to generate 4T1-Luc cells which lack human HER2/neu expression. 4T1-Luc and 4T1-HER2/neu-Luc provide mammary carcinoma cell lines with and without HER2/neu expression whose pulmonary metastases can be conveniently quantitated based upon luciferase activity from harvested lungs as well as counting lung nodules. Our Preliminary Studies demonstrate that when the primary tumor is resected 3 weeks after inoculation, 100% of the mice have readily detectable pulmonary metastasis when sacrificed as early as 6 weeks post-tumor implant. Without therapy, these pulmonary metastases grow to form gross metastatic nodules and kill the animal in 5-8 weeks. Our initial therapy trials will utilize groups of 10 animals who will receive tumor challenge with 4T1- HER2/neu-Luc followed by primary tumor resection at day 21. Forty-eight hours post-surgery, 10 animals will receive the HER2/neu vaccine program and 10 animals will receive control HIV-1 Gag vaccine by an identical schedule. Five weeks post-resection (8 weeks post-tumor implant), animals will be sacrificed with enumeration of lung tumor nodules and luciferase quantitated in lung homogenates as a quantitative estimate of tumor load. In separate studies, the time course of anti-micro-metastasis effects could be analyzed by sacrificing animals on the day of primary tumor resection and Q 2 weeks during vaccine therapy with quantitation of lung luciferase content in HER2/neu versus HIV-1 Gag treated animals; analysis of effector mechanisms can be carried our by treating groups of HER2/neu immunized animals with antibodies to selectively deplete CD4 or CD8 cells as previously described (9). As the therapy system evolves, additional experiments can include mouse survival data in addition to effects on pulmonary metastases and include additional controls, i.e., lack of effect of replicon HER2/neu on 4T1 which does not express HER2/neu.

The 4T1 cell line will only be used for immunotherapy experiments if successful immunoprotection against 4T1 cells expressing HER2/neu has been achieved (Specific Aim #3). As discussed under Specific Aim #3 Methods, if the 4T1- HER2/neu line is not able to function as a model for the anti- HER2/neu immune response,

we will employ the more immunogenic 4T07 line with human HER2/neu expression in subsequent immunotherapy trials. This would require intravenous injection of tumor cells to produce micro-metastases since the 4T07 line does not spontaneously metastasize (32). Additional problems could be encountered related to the time course of tumor growth with the 4T1 and 4T07 tumor lines. Both progress rapidly to macroscopic lung metastases which provides a therapeutic window of 4-6 weeks to address occult micro-metastases. As another alternative, we have the 66 tumor subline derived form the same parent murine mammary tumor as 4T1 and 4T07 (see chart on page A-3) readily available to us from F. Miller. Dr. Miller has shown that line 66 is immunogenic and produces pulmonary metastases either spontaneously or following intravenous injection which develop more slowly than 4T1 or 4T07.

These therapy experiments will utilize the best vaccine constructs, routes of administration, schedule and dose derived from the ongoing studies described in the Experimental Design sections for the first 3 specific aims. We believe we have reasonable alternative strategies if the 4T1 HER2/neu model has shortcoming but we would prefer to use this system since it closely resembles the human breast cancer problem.

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Advances in Brief

Characterization of Poliovirus Replicons Encoding Carcinoembryonic Antigen[1]

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Abstract

Recombinant vaccines hold great promise for the prevention and therapy of infectious diseases and cancer. We have explored the use of poliovirus as a recombinant vector to deliver genes into cells for the purpose of vaccination. For our studies, we have chosen to express the gene-encoding carcinoembryonic antigen (CEA) using a novel poliovirus vector. We have constructed a recombinant CEA-poliovirus replicon in which the CEA gene was substituted for the poliovirus capsid gene. Following *in vitro* transcription, the RNA was transfected into cells to demonstrate CEA expression. We found that a genome in which the region encoding the signal sequence of the CEA protein (amino acids 1–34) was removed was replication competent (*i.e.*, referred to as a replicon). We encapsidated the CEA-poliovirus replicon by transfecting this RNA into cells previously infected with a recombinant vaccinia virus (VV-P1) which expresses the poliovirus capsid protein (P1). Serial passage in the presence of VV-P1 resulted in the generation of stocks of these encapsidated replicons. Infection of cells with the encapsidated replicon containing the CEA-poliovirus genome resulted in expression of the CEA protein. To test immunogenicity, mice susceptible to poliovirus were given three doses of the encapsidated replicons via the i.m. route. By the third administration, a CEA-specific antibody response was detected. Potential future use of the poliovirus replicon system as both a parenteral and oral vaccine vector is discussed.

Introduction

The single-stranded RNA genome of poliovirus is approximately 7500 nucleotides in length (1). The translation product of the poliovirus genome is a long polyprotein translated from the entire coding region of the RNA genome (2, 3). The initial cleavage in the polyprotein between the P1 (capsid) and P2 (nonstructural) proteins is catalyzed by $2A^{pro}$ in a cotranslational reaction (4), whereas the other subsequent proteolytic cleavages are catalyzed by $3C^{pro}$ or a polyprotein containing $3C^{pro}$, designated as 3CD (5, 6).

The entire genome of poliovirus has been cloned, and the nucleic acid sequence has been determined (1, 7). The cDNA clone of poliovirus is infectious when transfected into cells (7). In recent years, interest has developed in using poliovirus as a vaccine vector because poliovirus replicates in many cell types, including gut epithelium and muscle (2). Since poliovirus is transmitted via a fecal/oral route, it is conceivable that the vaccine could be administered p.o. (8). Toward this goal, this laboratory has demonstrated that foreign gene sequences can be substituted into the poliovirus P1 region without affecting replication of the RNA as long as the translational reading frame was maintained (9). Since the replicons do not encode capsid proteins, they were encapsidated only when transfected into cells previously infected with a recombinant vaccinia virus (VV-P1) which expresses the poliovirus capsid precursor, P1 (10). Serial passage of these replicons in the presence of VV-P1 resulted in increasing titers of encapsidated replicons, allowing generation of stocks of the recombinant poliovirus vectors (10).

To further characterize poliovirus as a recombinant vaccine vector, we have constructed a recombinant poliovirus genome containing the 2.4-kilobase cDNA for CEA.[3] CEA is probably the most extensively characterized human tumor-associated antigen (11). The expression of CEA by adenocarcinoma cells is characteristic of human colon, breast, and non-small cell lung cancer, and has been proposed to be an effective target for tumor vaccine strategies (12–16). In this report, we describe the characterization and immunogenicity of replicons which express CEA for potential use in therapeutic strategies for breast and colon cancer.

Materials and Methods

Plasmid Constructions. All manipulation of recombinant DNA was carried out according to standard procedures (17). The starting plasmid for these studies, pT7-IC, contains the entire full-length poliovirus infectious cDNA positioned immediately downstream from the phage T7 promoter (9). The full-length cDNA encoding CEA, subcloned into a pGEM plasmid (18), was obtained from Dr. David Curiel, University of Alabama at Birmingham (originally obtained from Dr. Judy Kantor, NIH, Bethesda, MD).

For construction of the backbone poliovirus vector used for insertion of the CEA gene, two independent PCR reactions were performed. The first was used to amplify the region from nucleotides 1 to 743 of the poliovirus genome using the following PCR primers: 5′-CCA-GTG-AAT-TCC-TAA-TAC-GAC-TAC-CTA-TAG-GTT-AAA-ACA-GC-3′(5′ primer) and 5′-GA-TGA-ACC-CTC-GAG-ACC-CAT-TAT-G-3′(3′ primer).

A second set of PCR primers were designed to amplify a region of the poliovirus genome from 3370 to 6117. The PCR primers were designed so that a unique *Sna*BI restriction site would be created 12 nucleotides from the end of the *P1* gene, resulting in an additional four amino acids upstream from the tyrosine-glycine cleavage site. For subsequent subcloning, we digested the PCR product with *Sna*BI and *Bgl*II, which cuts at nucleotide 5601 in the poliovirus genome. The PCR primers used were as follows: 5′-CCA-CCA-AGT-ACG-TAA-CCA-CAT-ATG-G (5′ primer) and 5′-GTG-AGG-ACTG-CT-GG-3′(3′ primer).

The conditions for PCR were as follows: 1 min at 94°C, 3 min at 37°C, and 3 min at 72°C. After 30 cycles, a 7-min incubation at 72°C was included prior to cessation of the PCR reaction. PCR reactions were extracted successively with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1), and then DNA was precipitated with ethanol. After collection of the precipitate by centrifugation, the DNA was dried and resuspended in water. The DNA was then digested with the appropriate restriction endonuclease enzymes at the 5′ and 3′ ends of the PCR-amplified products.

Construction of pT7-IC-CEA-sig$^-$. To obtain a signal minus version of the CEA gene, PCR was used to amplify a region from the CEA cDNA. The primers used for this PCR reaction were as follows: 5′-CAC-CAC-TGC-CCT-CGA-GAA-GCT-CAC-TAT-TG-3′ (5′ primer) and 5′-CAC-CAC-TGC-CCT-CGA-GAA-GCT-CAC-TAT-TG-3′(3′ primer).

The DNA primers were chosen to create an *Xho*I site at the 5′ end and a

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; PCR, polymerase chain reaction; PFUs, plaque-forming units.

*Sna*BI site at the 3′ terminus of the amplified DNA. The length of the amplified DNA was approximately 100 base pairs less than that of the full-length amplified product for the CEA DNA, corresponding to a loss of 34 amino acids from the amino terminus representing the signal sequence. The conditions for PCR and isolation of the amplified product were as described in the earlier section. Prior to ligation, the amplified product was digested with *Xho*I and *Sna*BI.

The plasmid pT7-IC was digested with *Eco*RI and *Bgl*II. The DNA fragment which contains the poliovirus genome from nucleotides 5601 to the *Sal*I site (1.8 kilobases plus the 3.7 kilobases of the vector = 5.5 kilobases) was isolated. In the same ligation, this 5.8-kilobase fragment was ligated with the PCR-amplified products from nucleotides 1–743 (*Eco*RI-*Xho*I), the CEA gene (*Xho*I-*Sna*BI), and the PCR-amplified product containing poliovirus nucleotides 3370 (*Sna*BI) to 5601 (*Bgl*II). After incubation at 15°C overnight, the ligated products were transformed into *Escherichia coli* DH5α and the colonies were selected on ampicillin-containing plates. Plasmids isolated from individual colonies were screened for the desired insert by restriction enzyme digestion. The final plasmid was designated pT7-IC-CEA-sig$^-$.

Cell Culture and Viruses. HeLa cells were purchased from the American Type Culture Collection and were maintained in monolayer culture in DMEM (GIBCO/BRL) supplemented with 5% fetal bovine serum. BSC-40 cells were maintained in DMEM with 5% fetal bovine serum as described previously (19).

The vaccinia viruses used for these studies were grown in TK-143-B cells (American Type Culture Collection) and were concentrated for experimental use as previously described (19). The titers of vaccinia virus were determined by plaque assay on BSC-40 cell monolayers. The recombinant vaccinia virus used for the encapsidation experiments (VV-P1) was constructed as described previously (19). The recombinant vaccinia virus which expresses the carcinoembryonic antigen (rV-CEA) has been previously described (15, 16).

***In Vitro* Transcription, Transfections, and Metabolic Labeling.** *In vitro* transcription was carried out as described previously (9). The *in vitro* transcribed RNA was transfected into HeLa cells with DEAE-dextran (molecular mass, 500 kDa) as a facilitator as described previously (9). The cells were first infected with vaccinia virus for 2 h prior to transfection. After the 2-h infection period, the cells were washed once with DMEM without methionine-cysteine or leucine (depending on the metabolic label), and incubated in this medium for an additional 45 min to 1 h. In the case of replicon-infected cells, the infections were allowed to proceed 4–6 h prior to metabolic labeling. For [^{35}S]methionine-cysteine labelings, the cells were washed once and incubated in DMEM without methionine-cysteine plus [^{35}S]methionine-cysteine (Translabel; ICN) at 150 μCi/ml final concentration. In the case of metabolic labeling with [^{3}H]leucine, cells were labeled for 1.5 h using [^{3}H]leucine (Amersham) (350 μCi/ml) in a final volume of 0.2 ml leucine-free DMEM. After the labeling period, the cells were washed once with PBS and processed for radioimmunoprecipitation as described previously (19). To detect CEA protein, we used a CEA-specific monoclonal antibody (Col-1) at a concentration of 3 μg/ml.

Encapsidation and Serial Passage of Replicons by VV-P1. Procedures for encapsidation of the replicons have been described previously (10, 20). Briefly, HeLa cells were infected with 20 PFUs/cell of VV-P1 for 2 h. The cells were then transfected with *in vitro* transcribed RNA using DEAE-dextran (9). Sixteen h after transfection, the cells and medium were harvested by directly adding Triton X-100 to the medium, at a final concentration of 1%. The medium-cell lysate was clarified in a microcentrifuge for 20 min at 14,000 × *g*. The clarified lysate was treated with 20 μg/ml of RNase A at 37°C for 15 min, then diluted to 4 ml with 30 mM Tris-HCl (pH 8.0, 0.1 M NaCl, 1% Triton X-100), and overlaid on a 0.5 ml-sucrose cushion (30% sucrose, 30 mM Tris-HCl pH 8.0, 1 M NaCl, 0.1% BSA) in SW 55 tubes. The sucrose cushion was centrifuged at 45,000 rpm for 2 h. Pelleted material was washed with PBS-0.1% BSA and recentrifuged. The final pellet was resuspended in 0.6 ml complete medium. BSC-40 cells were infected for 2 h with 20 PFUs/cell of VV-P1, and 0.25 ml of the 0.6 ml was used to infect cells infected with VV-P1; after 24 h, the cells and media were harvested. This was designated Pass 1.

For serial passage of the encapsidated replicons, BSC-40 cells were infected with 20 PFUs of VV-P1/cell. At 2 h posttransfection, the cells were infected with Pass 1 of the encapsidated replicons. The cultures were harvested at 24 h postinfection by three successive freeze-thaws, sonicated, and clarified by centrifugation at 14,000 × *g* for 20 min. The supernatants were stored at −70°C or used immediately for additional passages, following the same procedure.

Estimation of the Titer of Encapsidated Replicons. Since the encapsidated replicons have the capacity to infect cells, but lack capsid proteins, they cannot form plaques and therefore virus titers cannot be quantified by traditional assays. To overcome this problem, we used a method to estimate the titer of the encapsidated replicons by comparison with wild-type poliovirus of known titer (10, 20). The resulting titer is then expressed in infectious units of replicons, since the infection of cells with the replicons does not lead to plaque formation due to the absence of the P1 capsid genes. We have determined experimentally that the infectivity of equal amounts of infectious units of encapsidated replicons correlates with equal amounts of PFUs of wild-type poliovirus.[4]

Immunization of Mice and Analysis of CEA-Specific Antibody Response. The encapsidated replicons contain a type I Mahoney capsid. Since the type I strain of poliovirus does not infect mice, we have made use of transgenic mice (designated as Tg PVR1) which express the receptor for poliovirus and are susceptible to poliovirus infection (21). Mice (4–5-week old) were immunized by i.m. injection at monthly intervals with poliovirus replicons expressing CEA; each mouse received 3 doses containing approximately 3 × 10^4 infectious units/mouse in 50 μl sterile PBS. To remove residual VV-P1, the replicon preparations were incubated with antivaccinia virus antibodies (Lee Biomolecular, San Diego, CA). The complete removal of residual VV-P1 was confirmed by the lack of vaccinia virus plaques after a 3-day plaque assay. Blood was collected from the tail veins of mice before and at selected times after immunization, centrifuged, and the plasma was collected and frozen until assay. ELISA was used for the determination of antigen-specific antibodies. The assays were performed in 96-well polystyrene microtiters plates (Dynatech, Alexandria, VA) coated with recombinant CEA or whole poliovirus type I at a concentration of 5 and 1 μg/ml, respectively. The CEA used for these studies was expressed in *E. coli*, using a pET vector with a 6-histidine affinity tag to facilitate purification (Novagen). The majority of the CEA product isolated from the nickel column used for purification was an 80-kDa protein corresponding to the nonglycosylated CEA. The poliovirus type I (Sabin) used was grown in tissue culture cells and purified by centrifugation (20). Dilutions of sera were incubated overnight at 4°C on coated and blocked ELISA plates, and the bound immunoglobulins were detected with horseradish peroxidase-labeled antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL). At the end of the incubation time (3 h at 37°C), the peroxidase substrate 2,2′-azino-bis-(3-ethylbenzthiazoline) sulfonic acid (Sigma, St. Louis, MO) in citrate buffer (pH 4.2) containing 0.0075% H_2O_2 was added. The color developed was measured in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA) at 414 nm. The results were expressed as absorbance values at a fixed dilution or as end point titration values.

Results and Discussion

Construction of Replicons Containing the Gene for CEA. The starting plasmid for these studies contains the full-length infectious poliovirus cDNA positioned downstream from a phage T7 promoter, designated pT7-IC (9) (Fig. 1*A*). After this plasmid is linearized at the unique *Sal I* restriction site, *in vitro* transcription mediated by phage T7 RNA polymerase is used to generate RNA transcripts. Transfection of the *in vitro* RNA transcript into tissue culture cells (*i.e.*, HeLa cells) results in translation and replication of the RNA, which leads to production of infectious poliovirus. From our previous studies, we have found that the infectivity of the RNA derived from this plasmid is in the range of 10^6 PFUs/μg transfected RNA (9). Previous studies have found that the majority of the P1 region of the poliovirus cDNA can be deleted without affecting the capacity of the resulting RNA genome to replicate when transfected into cells (22). To extend these studies, we have investigated whether the entire P1 region can be substituted with the 2.4-kilobase cDNA for CEA (Fig. 1*B*; Refs. 18 and 23).

In preliminary studies, we found that RNA containing the full-length

[4] D. C. Porter and C. D. Morrow, unpublished data.

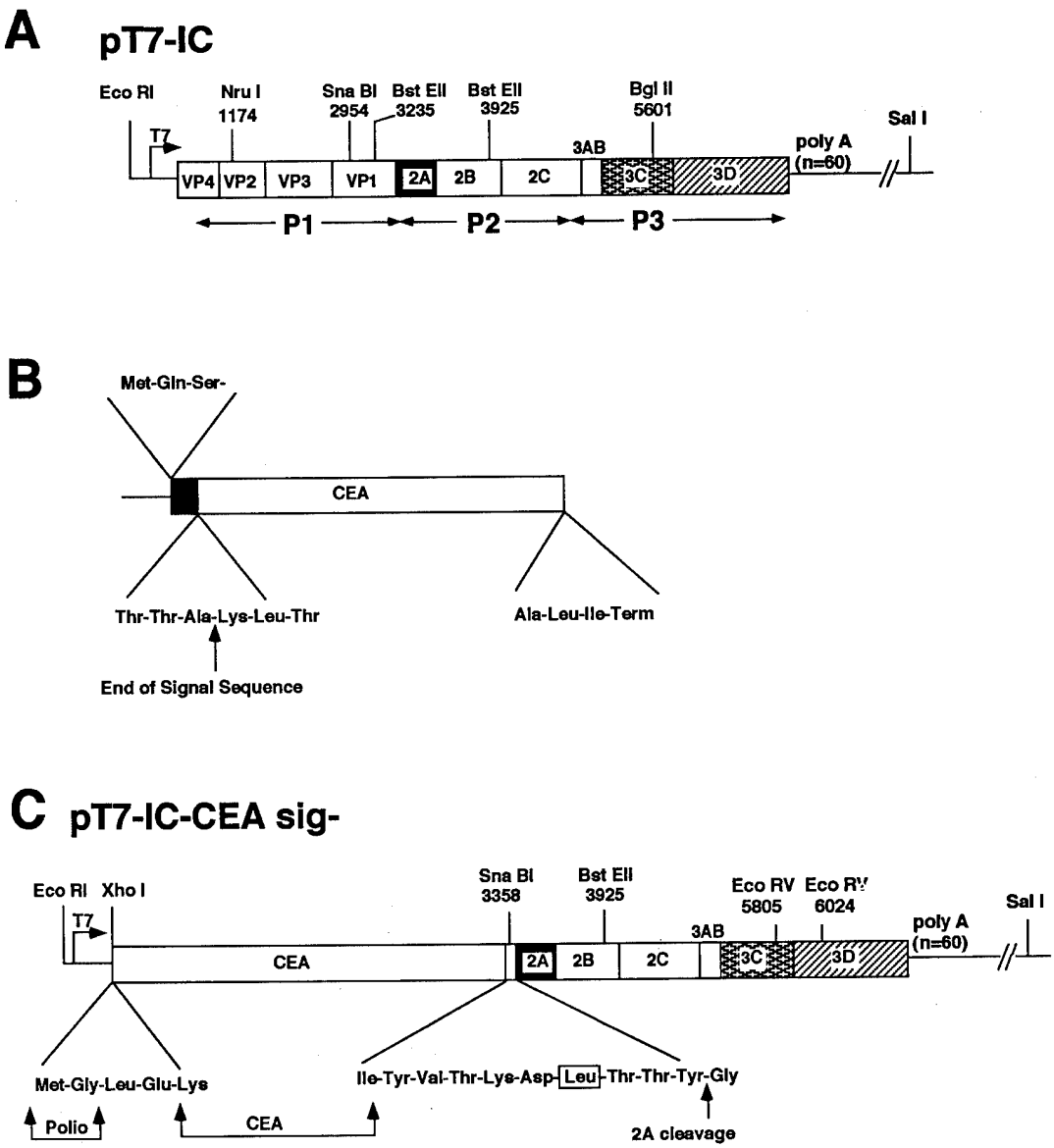

Fig. 1. CEA poliovirus replicons. In *A*, the full-length poliovirus infectious cDNA clone is depicted, with the coding regions for the viral proteins noted (1). The poliovirus capsid proteins (VP4, VP2, VP3, and VP1) are encoded in the P1 region of the poliovirus genome; the viral proteinase 2A and viral proteins 2B and 2C are encoded in the P2 region; and the viral proteins 3AB, 3C, and 3D (RNA polymerase) are encoded in the P3 region. The relevant restriction enzyme sites used for construction of the CEA recombinants are indicated. The *Sal*I restriction site is used to linearize the DNA template prior to *in vitro* transcription to generate the RNA transcripts used for transfection. *B*, schematic of the CEA protein. The signal sequence of the CEA protein consists of 34 amino acids (*black box*). The signal peptidase cleavage site occurs between the alanine and lysine amino acids. The codon for the carboxyl terminal isoleucine amino acid is followed by a TAA termination codon. *C*, construction of replicon containing the signal-minus CEA gene. PCR was used to amplify the CEA gene-encoding amino acids from the lysine at the amino terminus of signal-minus CEA to the isoleucine at the COOH terminus of CEA as shown in *B*. To subclone the gene encoding the signal-minus CEA protein, *Xho*I and *Sna*BI restriction endonuclease sites were positioned within the PCR primers. The final construct encodes the first two amino acids of the poliovirus P1 protein (Met-Gly) followed by two amino acids, leucine and glutamic acid (encoded by the *Xho*I restriction site), followed by the lysine amino acid of the signal-minus CEA protein. The CEA gene was positioned so that nine amino acids will be spaced between the C-terminal isoleucine of CEA and the tyrosine-glycine cleavage site for the 2A proteinase; the leucine amino acid required for 2A cleavage is *boxed*. This final construct was designated pT7-IC-CEA-sig$^-$.

CEA was not replication competent. It was possible that the signal sequence (amino acids 1–34) of the CEA protein was directing the CEA-P2-P3 fusion protein to the endoplasmic reticulum and in doing so prevented replication of the RNA. To test this possibility, we engineered the CEA gene to remove the first 34 amino acids of the CEA protein, which has been postulated to be the signal sequence (23, 24). We used PCR to amplify a region from amino acids 35–688 of the CEA gene that was then subcloned into the poliovirus replicon. The resulting DNA encoded the first two amino acids of the poliovirus P1 protein (Met-Gly), followed by two amino acids (Leu-Glu) derived from the *Xho*I restriction endonuclease site, followed by amino acid 35 (Lys) of the CEA protein. The isoleucine in CEA was fused to an additional nine amino acids (Tyr-Val-Thr-Lys-Asp-Leu-Thr-Thr-Tyr) in the predicted protein product. In this CEA protein, a leucine residue at the P4 position was included for optimal 2A autocatalytic cleavage (25).

Following *in vitro* transcription of pT7-IC-CEA-sig$^-$, the RNA transcripts were transfected into cells previously infected with VV-P1. For these studies we tested five independent clones containing the signal-minus CEA gene (designated as sig$^-$ CEA). As a positive control, we used a replicon which contains the HIV-1 *gag* gene (corresponding to the capsid, p24 protein) positioned between nucleotides 1174 and 2470 of the poliovirus genome. Cells were also infected with poliovirus to serve as a control in these experiments. In contrast to the results with the CEA replicons encoding the signal

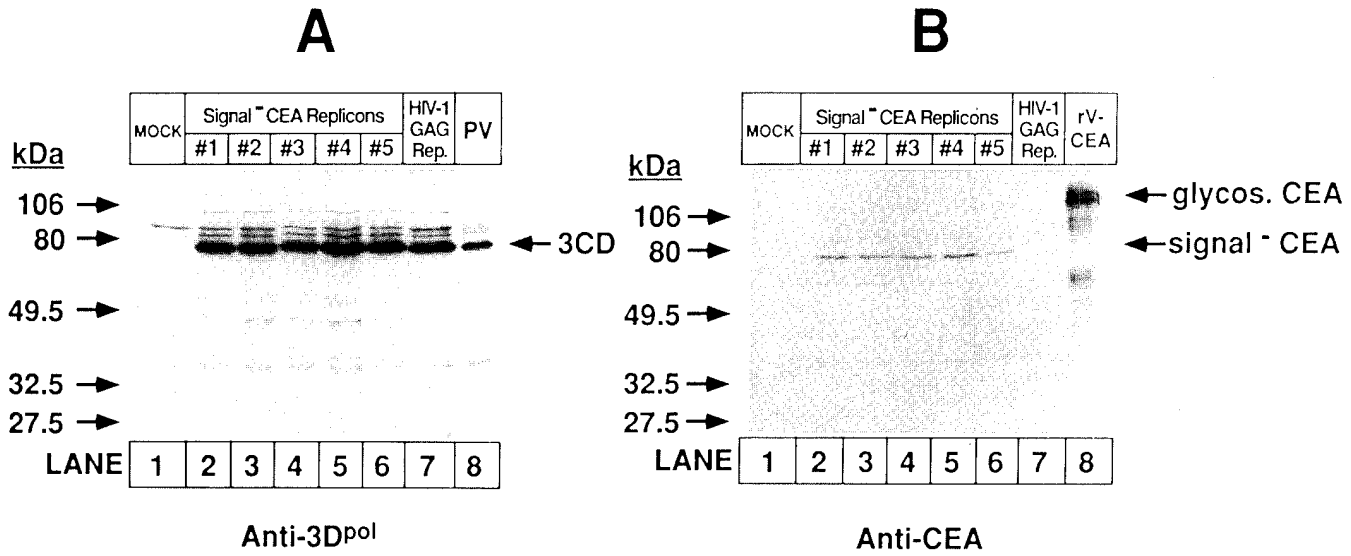

Fig. 2. Expression of the CEA protein in transfected cells. Five independent clones of pT7-IC-CEA-sig$^-$ were used for *in vitro* transcription reactions. The plasmid pT7-IC-Gag was used as a positive control (10). The RNA transcripts were transfected into cells previously infected with the recombinant vaccinia virus VV-P1. At 6 h posttransfection, the cells were metabolically labeled and ^{35}S-labeled proteins were immunoprecipitated with either anti-3D^{pol} (*A*) or anti-CEA (Col-1 monoclonal antibody, *B*). The immunoprecipitated proteins were separated on SDS-10% polyacrylamide gels, and autoradiograms of these gels are shown. Additional sets of cells were either infected with poliovirus (*A*) or a recombinant vaccinia virus which expresses CEA (*rV-CEA*, *B*) to serve as a source of marker proteins. The origin of the samples in each of the lanes for both panels is as follows: *Lane 1*, mock-transfected cells; *Lane 2*, cells transfected with RNA derived from clone 1 of pT7-IC-CEA-sig$^-$; *Lane 3*, cells transfected with RNA derived from clone 2 of pT7-IC-CEA-sig$^-$; *Lane 4*, cells transfected with RNA derived from clone 3 of pT7-IC-CEA-sig$^-$; *Lane 5*, cells transfected with RNA derived from clone 4 of pT7-IC-CEA-sig$^-$; *Lane 6*, cells transfected with RNA derived from clone 5 of pT7-IC-CEA-sig$^-$; *Lane 7*, cells transfected with RNA derived from transcription of pT7-IC-Gag 1; *Lane 8*, cells infected with either poliovirus (*A*) or rV-CEA (*B*). The migration of the molecular mass markers is noted. This migration of 3CD (*A*) and glycosylated and nonglycosylated forms of CEA (*B*) are also noted.

sequence, we detected the 3CD protein from cells transfected with RNA derived from five individual clones of pT7-IC-CEA-sig$^-$. The levels of 3CD expression in this experiment were comparable to those of cells transfected with RNA derived from *in vitro* transcription of pT7-IC-Gag 1, which was known from previous studies to be replication competent (Ref. 10; Fig. 2*A*). To determine if the CEA protein was expressed in the transfected cells, the lysates were also incubated with the Col-1 antibody to immunoprecipitate CEA-related proteins (Fig. 2*B*). Since the CEA protein should not be glycosylated, we expected that the CEA product would be approximately 80 kDa in molecular mass. In each of the transfections with RNA derived from the five independent clones, we immunoprecipitated an 80-kDa protein; this protein was not detected in cells transfected with replicons containing the HIV-1 *gag* gene.

Encapsidation and Serial Passage of Replicons Expressing CEA. We next determined whether the replicons containing the CEA sig$^-$ gene could be encapsidated if provided the poliovirus capsid proteins. In previous studies, we have demonstrated that replicons containing foreign genes could be encapsidated when serially passaged in the presence of a recombinant vaccinia virus (VV-P1) which expresses the poliovirus capsid precursor (20). Cells were infected first with VV-P1, followed by transfection with either the RNA derived pT7-IC-CEA-sig$^-$ or pT7-IC-Gag 1. A mock transfection was also included as an additional control. At 24 h posttransfection, extracts of the cells were generated by addition of detergents to the culture medium, and poliovirus-like particles were concentrated from the extracts by centrifugation through a 30% sucrose cushion. After resuspension, the concentrated material was used to infect cells that had been infected previously with either wild-type vaccinia virus or VV-P1 (passage 1). This coinfection was allowed to proceed overnight, after which extracts of the cells were generated by repeated freezing and thawing. The freeze-thaw extracts were clarified and used to repeat the coinfection procedure. This process was repeated for an additional nine serial passages to generate stocks of the encapsidated replicons. To determine whether the Pass 10 extracts contained encapsidated replicons, cells were infected with one quarter of the extracts and subsequently incubated with metabolic radiolabel at 6.5 h postinfection. The labeled proteins were immunoprecipitated with either anti-3D^{pol} or Col-1 antibody. No expression of 3CD protein was detected upon infection of cells with the sample originating from the mock-transfected cells and serially passaged 10 times with either wild-type vaccinia virus or VV-P1 (Fig. 3*A*). From analysis of 3CD expression, we concluded that RNA derived from transcription of pT7-IC-CEA-sig$^-$ was encapsidated when passaged in the presence of VV-P1, but not in the presence of wild-type vaccinia virus.

To determine if the CEA protein was expressed from the encapsidated replicons, we analyzed the extracts from infected cells that had been metabolically labeled followed by immunoprecipitation with the Col-1 antibody (Fig. 3*B*). Again, in samples from mock-transfected cells that had been subsequently passaged in the presence of either wild-type vaccinia virus or VV-P1, we detected no immunoreactive protein. A protein of molecular mass 80 kDa was immunoprecipitated from cells infected with the extracts originating from cells transfected with the RNA derived from pT7-IC-CEA-sig$^-$ which had been passaged in the presence of VV-P1, but not in the presence of wild-type virus. As expected, no Col-1 immunoreactive material was detected in cells infected with the RNA derived from pT7-IC-Gag 1, although this RNA was encapsidated in cells in the presence of VV-P1 (Fig. 3*A*).

Although the majority of the CEA protein immunoprecipitated from the cells infected with either stock of the encapsidated replicon RNA was the 80-kDa protein corresponding to the expected molecular mass of unglycosylated CEA, we noted that there was a small amount of protein immunoprecipitated corresponding to the molecular mass for the fully glycosylated CEA protein (180 kDa). To further explore this result, we used a concentrated stock of the signal-minus CEA replicon that had been passaged an additional 10 times (20 serial passages in all) and concentrated by pelleting through a 30% sucrose cushion prior to use in these experiments. Cells were infected with the encapsidated replicons, followed by metabolic radiolabeling for 1.5 h with [^{3}H]leucine since CEA contains more leucine amino acids than methionine or cysteine (23). This should increase the sensitivity of detection of the higher molecular mass CEA proteins. Three proteins

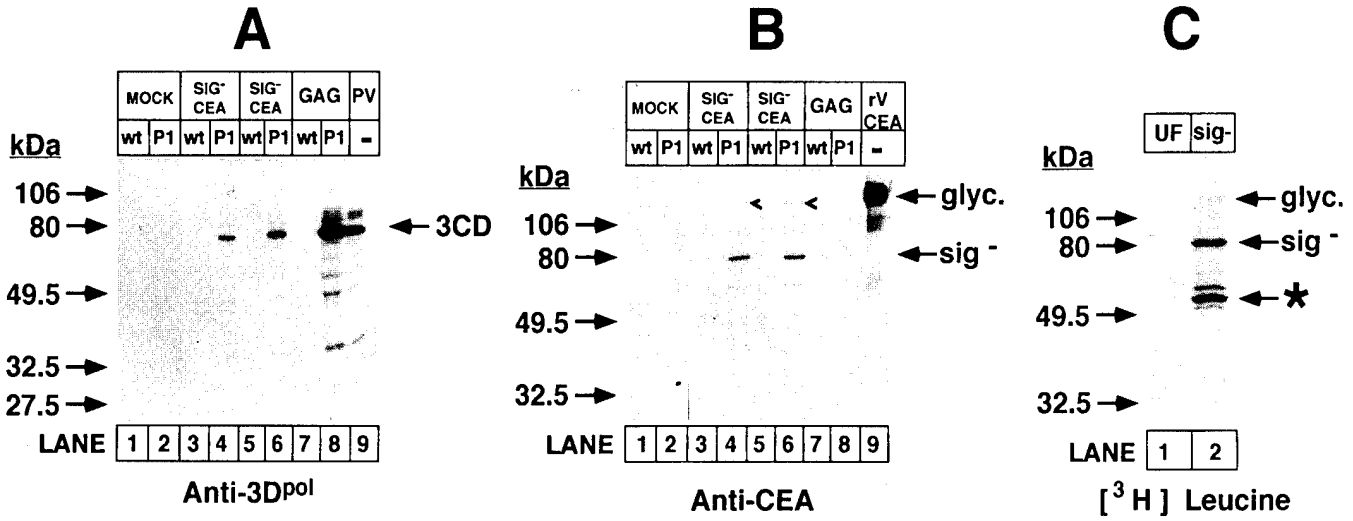

Fig. 3. Analysis of poliovirus and CEA-specific protein expression from cells infected with replicons which were encapsidated and serially passaged with capsid proteins provided by VV-P1. Cells were initially infected with VV-P1 at a multiplicity of infection of 20 and transfected with RNA derived from *in vitro* transcription of either pT7-IC-CEA-sig$^-$ or pT7-IC-Gag 1. The cells were harvested for isolation of encapsidated genomes as described in "Materials and Methods." The pelleted material was then used to infect cells previously infected with either wild-type VV or VV-P1. The encapsidated replicons were then serially passaged in this manner 10 times. For the experiment shown, the lysates from Pass 10 material were used to infect BSC-40 cells. At 6.5 h postinfection, the cells were starved for 30 min in methionine-cysteine-free DMEM, and then were metabolically labeled for an additional 90 min. The cell lysates were then analyzed by immunoprecipitation with either anti-3D^{pol} antibody (*A*) or antibody to the CEA protein (Col-1, *B*). The origin of the samples in the lanes for both panels is as follows: *Lane 1*, cells that were infected with wild-type vaccinia virus and then mock-transfected; *Lane 2*, cells that were infected with VV-P1 and then mock-transfected; *Lane 3*, cells that were infected with wild-type vaccinia virus and then transfected with RNA derived from *in vitro* transcription of pT7-IC-CEA-sig$^-$; *Lane 4*, cells that were infected with VV-P1 and then transfected with RNA derived from pT7-IC-CEA-sig$^-$; *Lane 5*, cells that were infected with wild-type vaccinia virus and then transfected with RNA derived from pT7-IC-CEA-sig$^-$ (a second independent clone); *Lane 6*, cells were infected with VV-P1 and then transfected with RNA derived from pT7-IC-CEA-sig$^-$ (a second independent clone); *Lane 7*, cells that were infected with wild-type vaccinia virus and then transfected with RNA derived from *in vitro* transcription of pT7-IC-Gag 1; *Lane 8*, cells that were infected with VV-P1 and then transfected with RNA derived from *in vitro* transcription of pT7-IC-Gag 1; *Lane 9*, cells that were infected with poliovirus (*A*) or recombinant vaccinia virus CEA (*rV-CEA*, *B*). The migration of the molecular mass markers is noted. In *A*, the migration of the 3CD protein is noted, whereas in *B*, the migrations of the glycosylated (*glyc.*) and nonglycosylated (*sig*$^-$) forms of CEA are noted. *Arrows*, position of the anti-CEA immunoreactive proteins of larger molecular mass observed in cells infected with encapsidated replicons containing the signal-minus CEA gene. In *C*, cells were infected with a Pass 20 stock of encapsidated signal-minus CEA replicons and then metabolically labeled with [^{3}H]leucine. *Lane 1*, uninfected cells metabolically labeled, followed by immunoprecipitation with Col-1 antibody; *Lane 2*, cells infected with encapsidated signal-minus CEA replicon, followed by immunoprecipitation with Col-1 antibody. The molecular mass standards are noted as well as the migration of glycosylated CEA (*glyc.*), nonglycosylated CEA (*sig*$^-$), and breakdown product (*asterisk*).

were immunoprecipitated using the Col-1 antibody from [^{3}H]leucine-labeled cells infected with the stock of the encapsidated replicon (Fig. 3*C*). One of these proteins corresponded to the unglycosylated protein with a molecular mass of approximately 80 kDa, while a protein of a smaller molecular mass, corresponding to approximately 52 kDa, was also immunoprecipitated. We believe that this protein represents a breakdown product of the CEA protein that was not detected previously because of the relatively few methionine or cysteine amino acids found in the CEA protein. A third protein of approximately 180 kDa was also immunoprecipitated, suggesting that glycosylated CEA protein might be produced in cells infected with the encapsidated replicons at low levels. The mechanism by which the signal-minus version of the CEA protein became glycosylated is under investigation. It is possible the signal-minus CEA still retains a signal sequence, albeit a poor one, as defined by von Heijne (26).

Immunogenicity of Encapsidated Replicons. To evaluate the immunogenicity of the encapsidated replicons which express the CEA protein, we used transgenic mice that express the receptor for poliovirus and are susceptible to infection with poliovirus (21). The mice were bred in a germ-free environment until use in the experiments. The four mice used in the experiment were bled prior to i.m. immunization with approximately 10^4 infectious units of the encapsidated replicon which expresses CEA. The serum samples from the mice at each of the pre- and postimmune time points were pooled and assayed using a solid-phase ELISA with whole poliovirus or recombinant CEA expressed in *E. coli* as the coating solution. By 28 days after the second booster immunization, a pronounced CEA-specific antibody response was detected as measured by the ELISA assay. The end point titer had increased from 1:25 (preimmune) to 1:6400 (Fig. 4*A*). A similar increase was observed in the antipoliovirus antibodies in the serum samples (Fig. 4*B*). As a control, we found no increase in anti-CEA antibodies in the sera from mice immunized with the replicon expressing HIV1 Gag (data not shown). Taken together, these results demonstrate that the replicons infect cells, presumably the muscle myofibers at the site of injection, and express sufficient amounts of CEA to stimulate an anti-CEA antibody response.

In this report, we have described the construction and characterization of RNA replicons which express the CEA protein when infected. A replicon encoding the signal-minus CEA protein was replication competent and expressed nonglycosylated CEA protein when transfected into cells. Using a recently described system to complement these defective genomes with a recombinant vaccinia virus expressing the poliovirus P1 protein (VV-P1), we generated stocks of encapsidated replicons containing the signal-minus CEA gene. The use of encapsidated poliovirus replicons as a vaccine vehicle has several distinguishing features. (*a*) This is one of the few vector systems based entirely on an RNA virus. Since poliovirus replication does not involve DNA intermediates, in contrast to retroviruses, the possibility of recombination in the host cell DNA is virtually eliminated. (*b*) Infection of cells with encapsidated replicons results in an amplification of the replicon RNA and preferential expression of the foreign gene over cellular gene products since poliovirus has evolved mechanisms to promote the synthesis of its own viral proteins (28). (*c*) It is important to stress that the encapsidated poliovirus replicons are noninfectious because they do not encode the viral P1 capsid proteins. The replicons require capsid proteins to be propagated and transmitted from cell to cell. Infection of cells or an animal with the encapsidated replicons alone then results in a single round of infection without chance

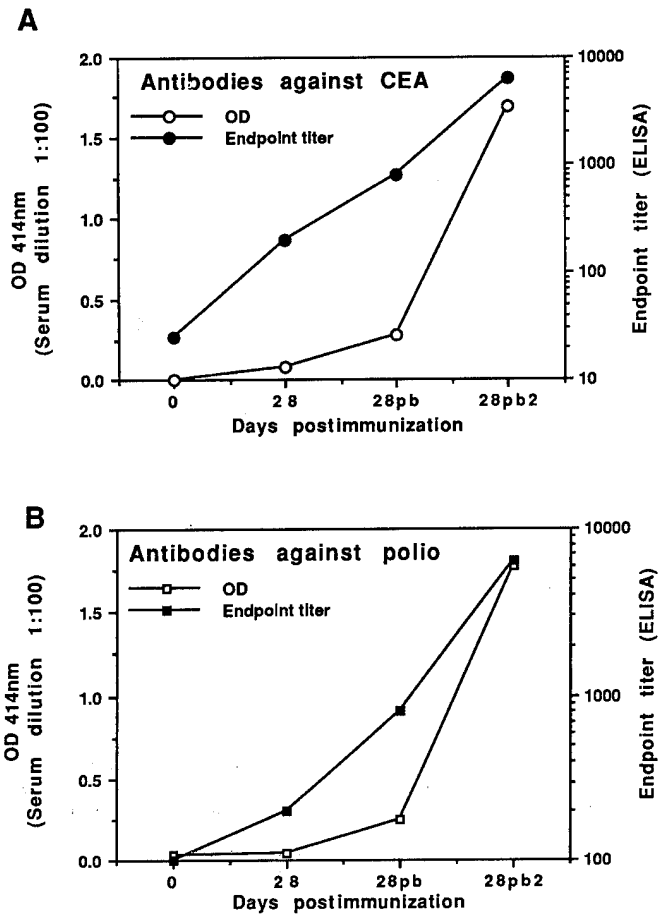

Fig. 4. Antibody response to encapsidated replicons expressing CEA. Four transgenic mice at 4–5 weeks of age were immunized with 3×10^4 infectious units of encapsidated replicon. The sera from each of the four animals at each time point were pooled and assayed for anti-CEA antibody using a solid-phase ELISA. The results are presented as absorbance 414-nm values at a fixed dilution and as end point titration values for anti-CEA (*A*) and antipoliovirus (*B*).

for further spread. Because of this feature, the encapsidated replicons can be exploited to deliver nucleic acids to cells without risk of viral spread.

The gene for CEA was chosen for our studies because of the practical importance of CEA in gene therapy approaches to antitumor vaccines (11, 12, 15, 24). It is clear from our studies that the encapsidated replicons expressing CEA were immunogenic when given i.m. to the transgenic mice. The availability of the encapsidated replicons which express CEA provides the opportunity for future evaluation of the characteristics of the immune response and possible antitumor effects. Current experiments are directed toward evaluation of the CEA replicons alone and in combination with other methods of delivery (*i.e.*, polynucleotide vaccination and recombinant vaccinia virus; Refs. 15 and 27). Since the replicons express a nonglycosylated CEA, it is possible the replicons alone, or in combination with other methods, might increase the immunogenicity of CEA. Finally, given the unique capacity of poliovirus to replicate in the gastrointestinal tract, we are exploring the possibility of using the encapsidated replicons expressing CEA for oral immunization to stimulate immunoreactive sites serviced by the mucosal immune system.

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