Polynucleotide Immunization of Nonhuman Primates against Carcinoembryonic Antigen

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ABSTRACT

In preparation for a Phase I trial of DNA immunization against carcinoembryonic antigen (CEA) in patients with colorectal carcinoma, we have produced a single plasmid DNA encoding CEA and hepatitis B surface antigen (HBsAg) under transcriptional regulatory control of two separate cytomegalovirus promoters within separate eukaryotic expression cassettes, designated pCEA/HBsAg. Hepatitis B surface antigen was included to provide an internal positive control for the efficacy of this immunization strategy with regard to the issue of breaking tolerance to a self-antigen. In the present work, we sought to examine the immunogenicity of this plasmid in a nonhuman primate model with close phylogenetic relationship to humans. Groups of pig-tailed macaques were immunized with pCEA/HBsAg by i.m. injection or particle bombardment of the skin according to a dose and schedule thought to be optimal for the respective technique of DNA immunization. Both administration techniques produced humoral and lymphoproliferative responses of comparable magnitude. However, delayed type hypersensitivity to CEA and CEA-specific interleukin-2 release were observed only in the i.m. group, suggesting a qualitative difference in the character of the immune response elicited by the two techniques of DNA immunization. The antibody responses to CEA and HBsAg were surprisingly persistent in that all immunized animals maintained moderate antibody titers against both antigens for more than 15 months after the last boost. No toxicity was observed during 2 years of follow-up, including no measurable levels of anti-DNA antibody. This antitumor immunization strategy is presently being examined in patients with metastatic colorectal carcinoma using pCEA/HBsAg administered by i.m. injection.

INTRODUCTION

There is increasing interest in the in vivo delivery of plasmid DNA encoding a relevant antigen as a novel approach to vaccination (1–4). Two emerging techniques for polynucleotide immunization are direct i.m. injection of plasmid DNA and delivery of plasmid DNA-coated gold beads by gene gun to the epidermal layer of the skin (5–7). Both techniques have elicited humoral and cellular immune responses to a variety of infectious agents including influenza and hepatitis B using a nonreplicating vector without adjuvant. The cutaneous particle bombardment strategy offers the potential advantage of antigen expression within an immunologically competent tissue (6). In this regard, the skin contains CD4-positive and CD8-positive T cells as well as epidermal Langerhans cells that function as highly potent antigen-presenting cells. Another potential advantage attributed to this strategy is the reported use of two or three orders of magnitude less DNA. Optimal immune responses have been observed with 0.4- to 4-μg doses of plasmid DNA by cutaneous bombardment (6, 7) compared with 50–100-μg doses by i.m. injection (1, 3, 8). Alternately, i.m. polynucleotide immunization offers the advantages of using a standard needle and syringe as well as the ability to escalate the dose of plasmid DNA without constraints related to the coating and delivery of beads.

Recent studies of polynucleotide immunization in nonhuman primates have demonstrated elicitation of measurable humoral and cytolytic T cell immune responses by i.m. administration of plasmid constructs encoding antigens derived from HIV-1, influenza A, herpes simplex virus, and hepatitis B virus (2, 3, 9–12). Other reports have described the induction of antigen-specific immunity against microbial antigens in primates after delivery of plasmid constructs by intradermal injection or gene gun-mediated cutaneous transfer (13, 14). Differences in the magnitude and character of immune responses generated by i.m. versus gene gun delivery of DNA vaccines in nonhuman primates are poorly understood. Gramzinski et al. (14) have reported that intradermal administration of a plasmid construct encoding Plasmodium yoelii circumsporozoite protein to Aotus monkeys produced quantitatively superior humoral immune responses to those generated by i.m. delivery of the plasmid construct. Recent data from Robinson’s group suggests enhanced immunogenicity of a polynucleotide vaccine construct encoding simian immunodeficiency virus antigens with multi-
ple-route (i.v., i.m., and gene gun) inoculations relative to plasmid delivery by gene gun alone (15).

We have reported previously the ability of i.m. polynucleotide immunization using a plasmid DNA encoding the full-length cDNA for human CEA under transcriptional regulatory control of the cytomegalovirus early promoter/enhancer to elicit CEA-specific humoral and cellular responses as well as protection against challenge with syngeneic, CEA-expressing colon carcinoma cells (8, 16). In preparation for a Phase I trial of i.m. DNA immunization against CEA in patients with colorectal carcinoma (17), we have produced a plasmid DNA encoding both CEA and hepatitis B surface antigen under transcriptional regulatory control of two separate CMV promoters within separate eukaryotic expression cassettes designated pCEA/HBsAg. HBsAg was included to provide an internal positive control for the efficacy of this immunization strategy without regard to the issue of breaking tolerance to a self-antigen.

The studies described in the present work were undertaken to evaluate issues pertaining to the use of this dual-expression plasmid in clinical trials. To this end, an animal model (pig-tailed macaque) with a close phylogenetic relationship to humans was used. Differences in the character and magnitude of antigen-specific immune responses after i.m. and cutaneous particle bombardment plasmid administration were investigated. To the best of our knowledge, this work is the first to accomplish the following: (a) a direct comparison of these two techniques of DNA immunization with regard to elicitation of humoral and cellular immune responses; (b) elicitation of immune response to two distinct antigens using a single plasmid DNA with two separate eukaryotic expression cassettes; and (c) elicitation of immune response via DNA immunization to a human tumor-associated self antigen in primates that share a highly homologous CEA molecule (18–20).

MATERIALS AND METHODS

Plasmid DNA for Vaccination. We obtained the cDNAs encoding full-length human CEA from J. Kanter, National Cancer Institute (21) as well as the small and middle (S2.S) proteins of hepatitis B surface antigen from Robert Whalen (Paris, France) (22, 23). These two cDNAs were inserted into a simplified eukaryotic expression vector that uses separate CMV intermediate-early promoter/enhancers to regulate transcription of CEA and HBsAg. The plasmid was derived from the commercially available eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). The plasmid contains two CMV intermediate-early promoter/enhancers and bovine growth hormone polyadenylation signals flanking polylinkers for insertion of heterologous open reading frames. The pcDNA3 plasmid was modified by deletion of the neomycin resistance gene, the ampicillin resistance gene, and nonessential viral sequences (24). The Tn903 kanamycin resistance gene from pUC4K (Pharmacia, Piscatway, NJ) was inserted to allow selective propagation in Escherichia coli. Fig. 1 shows a map of the resulting plasmid designated pCEA/HBsAg for the purpose of this report but was referred to previously as pGT63 (17).

E. coli strain DH5α (Life Technologies, Inc., Grand Island, NY) carrying the pCEA/HBsAg plasmid was grown in Terrific Broth (Life Technologies, Inc.) containing 50 µg/ml of kanamycin for selection. The plasmid DNA was purified using Qiagen Plasmid Mega kits (Qiagen Inc., Chatsworth, CA) according to the manufacturer’s instructions, with one Qiagen tip 2500 used for each liter of E. coli culture. Endotoxin was removed by extraction with Triton X-114 (Sigma Chemical Co., St. Louis, MO) as described previously (24).

Functional Validation of Plasmid DNA. The ability of pCEA/HBsAg to direct expression of CEA and HBsAg was confirmed by in vitro transfection of the C2C12 mouse myoblast cell line followed by immunoperoxidase staining for CEA using the human CEA-specific monoclonal antibody COL-1 (25) and analysis of cell-free supernatant for HBsAg by ELISA. Briefly, 2.5 µg of pCEA/HBsAg or a control plasmid encoding luciferase (pLuc) was mixed with DOTAP Liposomal Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) per the manufacturer’s instructions and incubated with 5 × 10^6 cells in a 3-cm tissue culture dish for 4 h. Cells then underwent media exchange, followed by an additional 48-h incubation. Cell-free supernatants were harvested and assayed for HBsAg by AUSAB EIA (Abbott Laboratories, Chicago, IL). The cells were analyzed for CEA expression by immunoperoxidase staining with the COL-1 monoclonal antibody. Myoblasts transfected with pCEA/HBsAg stained strongly for CEA expression and produced readily detectable levels of HBsAg in the supernatant, whereas cells transfected with irrelevant plasmid DNA (pLuc) produced negative results (data not shown).

Experimental Design. Groups of three pig-tailed macaques were immunized with plasmid DNA encoding CEA and HBsAg (pCEA/HBsAg) by i.m. injection or particle bombardment of the skin. Each group received the plasmid DNA according to a dose and schedule thought to be optimal for the respective technique of DNA immunization. Immunization via particle bombardment was performed according to the dose and schedule reported for primates by Haynes’ group at Auragen, Fig. 1 Plasmid encoding cDNAs for human CEA and the small and middle (S2.S) proteins of HBsAg within two separate expression cassettes. BGH, bovine growth hormone.
The preparation and immunization techniques for cutaneous bombardment-mediated immunization (13). Thus, in the present study, monkeys received pCEA/HBsAg at weeks 0, 10, 19, and 27 by particle bombardment. Monkeys in the i.m. injection group received 500 μg of pCEA/HBsAg into each quadriceps muscle for a total of 1 mg/time point at weeks 0, 3, 6, 9, 16, 25, and 33. This dose and schedule were selected based upon the available data from previous studies of i.m. DNA immunization in nonhuman primates (6, 13, 26) and our planned clinical trial (17). All animals were skin tested with native human CEA and recombinant HBsAg 6 weeks and 11 weeks after the last plasmid DNA immunization, respectively. Sera and whole blood for isolation of mononuclear cells were obtained at regular intervals for analysis of humoral and cellular immune responses to CEA and HBsAg. Twelve weeks after the last plasmid DNA immunization, complete blood counts and serum chemistries were obtained to evaluate any evidence of toxicity from the immunization protocol.

**Immunization of Monkeys.** Six female pig-tailed macaques (Macaca nemestrina), ages 2 to 3 years, were housed at the University of Alabama at Birmingham according to institutional and Animal Welfare Act guidelines. The macaques were assigned to two vaccination groups of three animals each. Monkeys in the first group received 500 μg of pCEA/HBsAg in 500 μl of normal saline inoculated into each quadriceps muscle using a standard needle and syringe for a total of 1 mg of plasmid DNA per time point administered at weeks 0, 3, 9, 16, 25, and 33. The second group of monkeys received 3-μg doses of pCEA/HBsAg by particle bombardment of the inguinal skin at weeks 0, 10, 19, and 27 as described below. Six weeks after the last plasmid DNA immunization, all animals received a single intradermal injection of native human CEA protein (ViroTech Diagnostics, Littleton, CO) as a skin test and to serve as a positive control mitogen; or baculovirus recombinant HIV gp160 as a control for proliferation induced by trace contaminants within the baculovirus recombinant CEA preparation. Control cells were cultured in a humidified atmosphere of 5% CO₂ in air for 6 days, followed by an overnight pulse with 1 μCi/well of tritiated thymidine. Cells were harvested with a Skatron automatic cell harvester, and incorporated radioactivity was quantitated using a solid-phase β-scintillation counter (Packard Matrix 9600; Packard Instrument Co., Downers Grove, IL). This solid-phase counter is ~3-fold less efficient than a liquid-phase scintillation counter such that raw cpm are 3-fold fewer than those seen with liquid counting. The mean cpm of quadruplicate wells correlates with the cellular proliferation rate. The stimulation ratio was calculated as mean cpm of the stimulated cells divided by mean cpm of the control cells. A positive response was defined as a postimmunization stimulation ratio of at least 2-fold greater than the preimmunization stimulation ratio for a given antigen.

**IL-2 Release.** Mononuclear cells were cultured exactly as above with the same panel of antigens or mitogens over the same range of concentrations with the exception that after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at −70°C. Supernatant IL-2 activ-
Table I  Antibody responses to CEA of monkeys immunized with pCEA/HBsAg

A. Particle bombardment animals

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Pre</th>
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<th>Week 13</th>
<th>Week 22</th>
<th>Week 30</th>
<th>Week 34</th>
<th>Week 107</th>
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<td>30,000</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>3,000</td>
<td>NT*</td>
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B. i.m. injection animals

<table>
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<tr>
<th>Monkey</th>
<th>Pre</th>
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<th>Week 19</th>
<th>Week 28</th>
<th>Week 36</th>
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<th>Week 113</th>
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<td>1,000</td>
<td>1,000</td>
<td>30,000</td>
<td>300</td>
</tr>
</tbody>
</table>

* Results are limiting dilution titers with values >0 representing a positive result.
* NT, not tested.

ity was quantitated based on the ability to support proliferation of CTLL-2 cells (27, 28). CTLL-2 cells were maintained in media containing 40% rat growth factor as a cytokine source prepared per American Type Culture Collection recommendations, and cells in log phase at least 48 h after the addition of fresh rat growth factor were selected for assay. CTLL-2 cells were washed five times in PBS to remove exogenous cytokines. These cells were added at 5000 cells/well in 100 μl of complete RPMI-10% FCS. Each well also received 100 μl of cell culture supernatant to be assayed for IL-2 activity. After 24 h in culture, cells were pulsed with tritiated thymidine (1 μCi/well) for 8 h and harvested with a Skatron automatic cell harvester, and incorporated radioactivity was determined using a Packard Matrix 9600 direct β-scintillation counter. Results were expressed as mean cpm of quadruplicate wells. The stimulation ratio was calculated as mean cpm of wells receiving supernatants from stimulated cells divided by mean cpm of wells receiving supernatants from control cells. A positive assay was defined as a stimulation ratio ≥2.0. Standard curves were also generated for each assay using recombinant human IL-2.

Anti-CEA Antibody Assay. Anti-CEA antibodies were quantitated by an ELISA. Microtiter plates were coated with 100 ng/well of baculovirus recombinant human CEA (MicroGeneSys, Meriden, CT). The plates were blocked with 1% BSA, 1% pig skin gelatin, and 3% nonfat dry milk in PBS for 1 h at room temperature, followed by washing. The plates were then incubated with various dilutions of monkey sera in PBS for 1 h at 37°C. Plates were washed, and antibody binding was detected with horseradish peroxidase-conjugated rabbit anti-monkey IgG antiserum (1:17,500; Sigma Chemical Co., St. Louis, MO), followed by a 30-min incubation with 100 μl of ABTS substrate (Sigma) in 0.05 M citric acid (pH 4.0). The reaction was stopped by the addition of 100 μl of 0.16 M oxalic acid, and the absorbance was read at 405 nm. Serum titers of anti-CEA antibody were calculated as the limiting dilution of serum producing an absorbance greater than 0.100 and at least 3-fold greater than the absorbance of autologous preimmune serum at the same dilution.

Competitive Inhibition. For competitive inhibition assays, dilutions of immunized monkey sera in PBS were selected that produced absorbance values of 0.8–1.5 in the ELISA for anti-CEA antibody. These diluted sera were preincubated for 1 h at 37°C with 10 μg/100 μl of baculovirus recombinant human CEA, native human CEA purified from human colon cancer liver metastases (Vitro Diagnostics), BSA as a negative control for nonspecific protein inhibition, or no inhibitor. The diluted sera with or without inhibitor protein were then added to microtiter plates coated with baculovirus recombinant CEA, and the remainder of the ELISA for anti-CEA antibody was conducted as described above. Preincubation of immune sera with an excess of the baculovirus recombinant CEA used to coat the plates uniformly reduced the ELISA absorbance by >90% and defined the background absorbance due to nonspecific binding. The percentage of inhibition of absorbance was calculated as follows:

\[
\frac{A - B}{A - C} \times 100
\]

where \(A\) is the absorbance produced by serum alone, \(B\) is the absorbance produced by serum preincubated with an experimental inhibitor (BSA or native human CEA), and \(C\) is the background absorbance due to nonspecific binding observed with serum preincubated with the recombinant CEA used to coat the plate.

DTH Testing. DTH responses were examined by skin testing with native human CEA 6 weeks after the last plasmid DNA immunization. Native CEA purified from human colon cancer liver metastases (Vitro Diagnostics) at 150 μg in 0.1 ml of PBS was injected intradermally into each of the six macaques immunized with pCEA/HBsAg as well as five age-matched, naive pig-tailed macaques. Induration and erythema were measured with calipers 48 h later. Punch biopsies were taken of positive responses, and the DTH nature of the reaction was confirmed by histopathological examination.

RESULTS

Antibody Response To CEA. Anti-CEA antibody responses were evaluated in monkeys immunized with pCEA/HBsAg by ELISA assay with results provided in Table 1. All animals were negative for anti-CEA antibody preimmunization. One of three monkeys in the particle bombardment group dem-
protein inhibition reduced binding by only 1–6%. Conversely, prior to skin testing with native human CEA protein and results
respectively inhibit binding of the monkey antisera to wells coated
or insect cell contaminants within the protein preparation. For
nant human CEA represented binding to CEA and not trace viral
protein. The antibody response to CEA was surprisingly durable in
caques showed no evidence of antibody responses to CEA 1
fold increase in anti-CEA antibody titer was observed in all
protein 6 after the last DNA immunization served as a boost for
comparable. Intradermal skin testing with native human CEA
body responses to CEA, and the titers of the two groups were
incremental regarding the
two techniques of DNA immunization regarding the
preincubation with excess native human CEA inhibited binding by
85–98%. These results indicate that the monkey antisera recognize epitopes shared between native human CEA and baculovirus recombinant CEA rather than trace contaminants in the protein preparation. This result was expected because the animals were immunized with plasmid DNA and thus were never exposed to the recombinant protein to allow development of an antibody response to trace contaminants.

**Antibody Response to HBsAg.** Anti-HBsAg antibody responses were evaluated in monkeys immunized with pCEA/ HBsAg by the commercially available AUSAB ELISA (Abbott Laboratories, North Chicago, IL), which detects antibodies to the small S protein of HBsAg. All animals were negative for anti-HBsAg antibody preimmunization. One of three monkeys in each group developed an antibody response to HBsAg 13–19 weeks after primary immunization (Table 2). Three weeks after the last DNA immunization, only one of three animals in the particle bombardment group was positive for antibodies to HBsAg, whereas all three animals in the i.m. group were positive. Three weeks after an intradermal boost with recombinant HBsAg protein, all five available animals had detectable antibody responses to HBsAg. As described in “Toxicology,” monkey no. 3 died prior to week 41 of an unrelated cause. Anti-HBsAg antibody responses $\geq 10$ mIU/ml in the Abbott ELISA correlate with protective immunity in humans. By this
criterion, all five animals developed a protective level of antibodies 3 weeks after the protein boost. Unpublished data from Smith Kline Beecham Pharmaceuticals have shown that the geometric mean titer of anti-HBsAg antibody was $< 10$ mIU/ml among more than 700 human subjects 1 month after a single immunization with the recombinant HBsAg protein vaccine. This suggests that, although DNA immunization did not elicit a detectable antibody response to HBsAg in monkey no. 1, it successfully primed the antibody response. The antibody response to HBsAg was surprisingly persistent in that 69 weeks after a single protein boost, all five animals maintained a high level of antibodies. Anti-HBsAg antibody titers increased in two of three animals in the i.m. group during the 69-week rest without further immunization.

Because the plasmid DNA encodes both the pre-S2 and S regions of HBsAg, it is possible that the Abbott ELISA, which only measures antibody to the S region, underestimated the antibody response to HBsAg. To examine this possibility, representative sera from all six monkeys were assayed by the Monolisa Anti-HBsAg kit (Sanofi, Quebec, Montreal, Canada), which detects antibody to both the pre-S2 and S regions. The results did not differ significantly from those obtained with the Abbott ELISA (data not shown).

**Lymphoproliferative Response to CEA.** The lymphopro
ductive responses to CEA elicited by DNA immunization are illustrated in Fig. 3. Monkeys nos. 1 and 2 in the particle
bombardment group demonstrated lymphoproliferative responses to CEA 3 weeks after primary immunization, which increased in magnitude 9 weeks after primary immunization. Both animals continued to demonstrate lymphoproliferative responses to CEA 16 weeks after DNA immunization was initiated and 11 weeks after a boost with CEA protein (week 44). The third monkey in this group failed to develop a lymphoproliferative response to CEA.
Table 2. Antibody responses to HBsAg of monkeys immunized with pCEA/HBsAg

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Pre</th>
<th>Week 13</th>
<th>Week 22</th>
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<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NT‡</td>
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</table>

<table>
<thead>
<tr>
<th>Monkey</th>
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<td>102</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>91</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

‡ Values are mIU/ml of antibody to HBsAg as quantitated by the commercially available Abbott ELISA with values >10 correlating with protective immunity in humans.

All three monkeys in the i.m. immunization group developed lymphoproliferative responses to CEA by 16 weeks after primary immunization. The lymphoproliferative responses observed 6 weeks after the week-16 plasmid DNA boost were variable with monkey no. 4 boosting well, monkey no. 6 remaining positive but with decreased magnitude of response, and monkey no. 5 becoming negative. All three animals demonstrated persistent lymphoproliferative responses to CEA 11 weeks after a CEA protein boost (week 50).

To demonstrate the specificity of the observed lymphoproliferative responses, Table 3 provides data from a representative assay for each monkey. All animals except no. 3 demonstrated proliferative responses to baculovirus recombinant human CEA, with stimulation ratios ranging from 3.3 to 44. All monkeys failed to respond to BSA included as a control antigen, with stimulation ratios ranging from 0.5 to 1.3. Furthermore, all animals failed to respond to baculovirus recombinant HIV gp160 included as a control for potential recognition of trace baculovirus and insect cell contaminants in the CEA protein preparation. Stimulation ratios to baculovirus gp160 ranged from 0.9 to 1.9. Mitogen responses were brisk from all animals.

IL-2 Release. Peripheral blood mononuclear cells obtained from each monkey at a variety of time points were cultured for 3 days with a panel of antigens or mitogens, and the cell-free supernatants were assayed for IL-2 release as described in “Materials and Methods.” Despite IL-2 release in response to PHA, all three monkeys in the particle bombardment group as well as monkeys nos. 5 and 6 in the i.m. group showed no detectable IL-2 release in response to CEA at any time point. However, monkey no. 4 in the i.m. injection group did demonstrate CEA-specific IL-2 release on multiple occasions, with results shown in Table 4. Monkey no. 4 showed no evidence of CEA-specific IL-2 release preimmunization or 3 weeks after the primary immunization. However, IL-2 release was readily detected in response to CEA after the first, third, fourth, and fifth boosts, with values ranging from 18–86 mIU/ml of IL-2 in the cell culture supernatant. No IL-2 release was observed from cells cultured in media alone or with BSA as a control antigen.

DTH Test. Monkeys were tested for DTH responses to native human CEA 6 weeks after the last plasmid DNA immunization. In parallel, five age-matched, naive pig-tailed macaques were tested with CEA as controls. As shown in Fig. 4, no evidence of erythema or induration was observed in any of the naive control monkeys or any of the monkeys immunized by
CEA. Monkey no. 4 developed a 10-mm area of induration with associated erythema but no necrosis. Monkey no. 6 developed a 59-mm area of induration with associated erythema and central necrosis requiring surgical excision. Punch biopsies were obtained from the responders, and the DTH nature of the response was confirmed by histopathological examination. All monkeys were also tested for DTH responses to yeast-derived recombinant HBsAg protein 11 weeks after the last plasmid DNA immunization with negative results (data not shown).

Toxicology. There was no evidence of fever, weight loss, regional lymphadenopathy, hepatomegaly, or splenomegaly in any of the monkeys. Animals were tested for complete blood counts, differentials, and hepatic and renal chemistries 12 weeks after the last plasmid DNA immunization. Complete blood counts and differential counts were entirely within normal limits. Specifically, no evidence of granulocytopenia was observed after elicitation of humoral and cell-mediated immune responses to human CEA, despite the fact that simian granulocytes have been shown to express normal cross-reacting antigen (29). Hepatic function was assessed by measuring serum albumin, bilirubin, transaminases, y-glutamyl transpeptidase, and alkaline phosphatase. These values did not differ from normal standards with the exception of mildly elevated transaminases in monkey no. 3. Four days after these blood samples were obtained, monkey no. 3 was found dead in its cage without preceding signs of illness. Necropsy revealed that the cause of death was acute gastric dilation and torsion, which is common among nonhuman primates. Renal function was assessed by measuring blood urea nitrogen and serum creatinine levels, which were all within normal ranges. Serum creatine kinase levels were obtained to look for evidence of possible myositis, particularly among the monkeys in the i.m. injection group that had received 1-mg doses of plasmid DNA on seven occasions over an 8-month period. Although we were unsuccessful in finding a published normal range for creatine kinase in pig-tailed macaques, the mean level for animals in the i.m. injection group was lower than the mean for animals in the particle bombardment group in which myositis would not be expected to occur. The mean creatine kinase levels ± SE for the i.m. and particle bombardment groups were 423 ± 128 and 805 ± 101, respec-

| Table 3 | Specificity of the lymphoproliferative responses of monkeys immunized with pCEA/HBsAg |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Monkey         | Weeks after primary immunization | Media          | CEA             | gp 160<sup>a</sup> | BSA<sup>b</sup> | PHA             |
| 1              | 9                | 170 ± 40<sup>c</sup> | 1,700 ± 300     | 220 ± 30         | 160 ± 10        | 21,000 ± 1,000  |
| 2              | 9                | 150 ± 20         | 5,900 ± 700     | 130 ± 20         | 200 ± 50        | 30,000 ± 2,000  |
| 3              | 9                | 200 ± 30         | 350 ± 20        | 260 ± 30         | 210 ± 40        | 19,000 ± 2,000  |
| 4              | 22               | 62 ± 7           | 2,700 ± 200     | 76 ± 9           | 57 ± 4          | 21,000 ± 1,000  |
| 5              | 16               | 110 ± 10         | 360 ± 30        | 100 ± 10         | 120 ± 20        | 28,000 ± 2,000  |
| 6              | 16               | 96 ± 20          | 3,800 ± 200     | 180 ± 30         | 51 ± 10         | 22,000 ± 4,000  |

<sup>a</sup> Baculovirus recombinant HIV gp160 protein.  
<sup>b</sup> Bovine serum albumin.  
<sup>c</sup> Values are mean cpm of quadruplicate wells ± SE.

| Table 4 | Mononuclear cell IL-2 release after immunization with pCEA/HBsAg<sup>ac</sup> |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Stimulus        | Pre             | Week 3          | Week 6          | Week 16         | Week 22         | Week 28         |
| Media alone     | 0±1            | 0±1             | 0±1             | 0±1             | 0±1             | 0±1             |
| Bovine serum albumin | 0±1        | 0±1             | 0±1             | 0±1             | 0±1             | 0±1             |
| CEA             | 0±1            | 0±1             | 86 ± 7          | 42 ± 1          | 56 ± 5          | 18 ± 1          |

<sup>a</sup> Peripheral blood mononuclear cells were obtained from monkey no. 4 during the course of immunization with pCEA/HBsAg by i.m. injection and assayed for CEA-specific IL-2 release.  
<sup>b</sup> Values are mIU/ml of IL-2 in cell culture supernatant with values >0 representing a positive result. Values >0 are reported as the mean ± SE for triplicate determinations.

Fig. 4. DTH skin test responses to CEA protein. Monkeys were tested for DTH responses to native human CEA 6 weeks after the last plasmid DNA immunization. Five naive monkeys simultaneously challenged with CEA in the same fashion served as controls. Results represent the area of induration 48 h after the intradermal dose of CEA protein in each individual animal in the naive, particle bombardment, and i.m. injection groups.
tively. Furthermore, the creatine kinase levels of animals in the i.m. group were within the normal range for the closely related rhesus macaque (77–790 units/liter).

One week after the last DNA immunization, serum samples from all six monkeys were assayed for anti-double-stranded DNA antibodies by a commercial assay available from Sanofi Diagnostics (Chaska, Minnesota) with negative results. This assay uses a polyclonal anti-human IgG antisera for detection. This assay was repeated, substituting rabbit polyclonal anti-monkey IgG antisera (Sigma, St. Louis, MO), and still no monkey showed evidence of anti-double-stranded DNA antibodies (data not shown). The five surviving monkeys from this study have remained alive and well without signs of illness for more than 1 year since completion of the vaccination protocols.

DISCUSSION

Limited information is available regarding the efficacy of polynucleotide immunization in nonhuman primates. Liu and co-workers (2, 11) have demonstrated antibody responses to influenza proteins after i.m. injection of a mixture of five plasmids encoding distinct influenza antigens using doses as low as 10 μg of each construct. The same group has also shown induction of cytolytic T-cell responses after i.m. injection of a plasmid encoding HIV-1 env at a 200-fold higher dose of 2 mg (11, 30). Gramzinski et al. (14) was unable to demonstrate antibody responses to Plasmodium yoelii circumsporozoite protein after four 2-mg doses of the antigen-encoding plasmid delivered i.m. over 21 weeks (14). Prince et al. (12) observed transient antibody responses and protection against viral challenge after i.m. injection of 1-mg doses of plasmid DNA encoding HBsAg in newborn chimpanzees (12). Wang et al. (9) and Ugen et al. (31) have demonstrated humoral and cellular immune responses elicited by 100-μg doses of a plasmid encoding HIV-1 env administered by i.m. injection with bupivacaine (9, 31). Thus, there appears to be considerable variability in the dose of plasmid DNA required to elicit humoral immune responses by i.m. polynucleotide immunization, perhaps depending upon the immunogenicity of the encoded antigen, age, and/or the primate species used. It is therefore somewhat surprising that we observed antibody responses to CEA in nonhuman primates that share a highly homologous molecule, whereas Gramzinski et al. (14) saw no immune response to a malaria antigen.

Information regarding the efficacy of DNA immunization by particle bombardment in nonhuman primates is even more limited. The group from Auragen, Inc., developers of the Acell® gene gun, immunized rhesus macaques with 4-μg doses of plasmid DNA encoding SIV env on weeks 0, 12, and 30, a dose and schedule very similar to the one that we used with pCEA/HBsAg. They reported antibody titers 3–10-fold higher than those achieved using a schedule of six doses over 30 weeks, but no cellular immune response data were presented (13). To the best of our knowledge, this report is the first to provide lymphoproliferative response, lymphokine release, and DTH data after DNA immunization of nonhuman primates by either particle bombardment or i.m. injection without bupivacaine.

We have previously compared immunization with plasmid DNA encoding CEA by i.m. injection versus cutaneous particle bombardment in mice using the same schedule and comparable doses. Both administration techniques produced similar CEA-specific lymphoproliferative and anti-CEA antibody responses. However, only i.m. injection provided immunoprotection against challenge with syngeneic, CEA-expressing colon carcinoma cells (32). The anti-CEA antibody response elicited by particle bombardment was exclusively of IgG1 isotype compatible with a T helper 2 immune response, whereas i.m. injection produced a substantial IgG2a response compatible with T helper 1 activation (33, 34). These results suggest that particle bombardment elicits an immune response to CEA that is qualitatively different from that produced by the same DNA administered by i.m. injection with resultant differences in tumor protection.

Similar to our studies in mice, both techniques of administration of pCEA/HBsAg in nonhuman primates elicited anti-CEA antibody responses of similar magnitude in all immunized animals that boosted within 1 week of a single CEA protein exposure. Similarly, i.m. injection of plasmid DNA elicited antibody responses to HBsAg in all three monkeys prior to the protein boost. However, particle bombardment elicited an anti-HBsAg antibody response in only one of three animals prior to protein exposure. There was no significant difference between the two immunization techniques regarding the durability of antibody responses in that all immunized animals maintained moderate antibody titers against both CEA and HBsAg for over 15 months after the last boost. Both immunization techniques generated CEA-specific lymphoproliferative responses of similar magnitude observed at multiple time points in all three animals inoculated i.m. and two of three animals in the particle bombardment group. However, DTH compatible with a T helper 1 response was observed only in the i.m. group. Furthermore, CEA-specific release of interleukin-2, a T helper 1 cytokine, was only observed in the i.m. group, albeit in only one of three animals. This is suggestive evidence that immunization with plasmid DNA encoding CEA by i.m. injection favors development of a T helper 1 response thought to be more important for antitumor immunity in both mice and monkeys, whereas epidermal delivery by gene gun may favor T helper 2 activation. The differences in dose and schedule selected in an effort to optimize immunization by the i.m. and epidermal gene gun routes may have contributed to the observed differences in immune responses between the two groups in the primate study. Based on this data, we have chosen to examine i.m. administration of pCEA/HBsAg in an initial clinical trial of this antitumor immunization strategy in patients with colorectal carcinoma (17).

We were impressed by the antibody response to human CEA given the fact that monkeys have a CEA molecule that is highly homologous to that found in humans (18–20). We therefore confirmed the specificity of this reactivity by competitive inhibition studies with human CEA extracted from human colon cancer metastases. These antibodies were capable of mediating antibody-dependent, cell-mediated cytotoxicity with human effector cells (date not shown).

Both techniques of immunization with pCEA/HBsAg were free of toxicity. There was no evidence of inflammation or tenderness at the inoculation sites. Analysis of complete blood counts and serum chemistries revealed no significant abnormal-
ities. Likewise, there was no evidence of myositis, despite animals in the i.m. group receiving a total of 7 mg of plasmid DNA over 33 weeks. Previous authors have cited the potential formation of anti-DNA antibodies with resultant autoimmune disease as a major safety issue posed by nucleic acid vaccination (35). Consistent with the findings of other groups (11, 36), none of the animals immunized had measurable anti-DNA antibodies.

The anti-CEA antibody responses required ~20–30 weeks to reach moderate titers after primary Immunization of monkeys with pCEA/HBsAg by either delivery technique. Lymphoproliferative responses occurred within 3–9 weeks of primary immunization in the gene gun group but were not observed until week 16 in the i.m. group. Thus, DNA immunization would seem poorly suited for use as therapy for cancer patients with overtly metastatic disease. Such unfortunate patients typically have limited life expectancy, prohibiting completion of a protracted course of immunizations. Therefore, we believe the most promising application of DNA immunization technology to cancer therapy will be in the adjuvant setting in an attempt to eradicate micrometastases and thus prevent or delay tumor recurrence.

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