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Immune Response in Mice following Immunization with DNA Encoding Fragment C of Tetanus Toxin

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Tetanus toxin is a potent neurotoxin synthesized by *Clostridium tetani*. Immunization with fragment C protein, the nontoxic C-terminal domain of tetanus toxin, will protect mice against lethal challenge with tetanus toxin. A synthetic gene encoding fragment C (*tetC*) had previously been shown to express high levels of fragment C in *Saccharomyces cerevisiae*. A plasmid, pcDNA3/tetC, which encodes the synthetic *tetC* gene expressed under the control of the human cytomegalovirus major intermediate-early promoter/enhancer region, was constructed. Expression of fragment C was observed in eukaryotic cells growing in vitro following transfection with pcDNA3/tetC. The immune response induced by intramuscular immunization with pure pcDNA3/tetC DNA was evaluated in a murine model. Anti-fragment C serum immunoglobulin and proliferative responses in splenocytes were observed in BALB/c mice following two immunizations with pcDNA3/tetC. The major immunoglobulin G subclass that recognized fragment C was immunoglobulin G2a, and the stimulated splenocytes secreted high levels of gamma interferon. Immunity to tetanus is dependent on the presence of neutralizing serum antibodies against tetanus toxin. Sufficient anti-fragment C serum immunoglobulins were induced by DNA-mediated immunization to protect mice against lethal challenge with tetanus toxin.

Since the observation that an immune response can be induced by direct immunization with plasmid DNA (38), several nucleic acid vectors encoding foreign antigens have been evaluated as vaccines. Nucleic acid vaccination has been most extensively studied with influenza virus antigens. Protective immune responses have been induced in mice (19, 23, 41), ferrets (43), and chickens (18, 19) following intramuscular or epidermal immunization with plasmid vectors encoding the influenza virus hemagglutinin or nucleoprotein. Significantly, recent work has shown that vaccination with a combination of plasmid DNAs encoding influenza virus A hemagglutinin or nucleoprotein is more effective in protecting ferrets against challenge than the contemporary human influenza virus A vaccine (14). Thus, DNA-mediated immunization appears to induce both humoral and cell-mediated responses against the influenza virus antigens. Immune responses against viruses (9, 12, 24, 29, 42, 47, 50), parasites (37, 48, 49), and tumor cells (7, 8) have also been induced by DNA-mediated immunization. However, the capacity of nucleic acid vaccines to induce protective immune responses against bacterial antigens remains largely undetermined (1, 26).

Tetanus toxin is a potent neurotoxin synthesized by *Clostridium tetani*. At present, vaccine preparations against tetanus are produced by formaldehyde inactivation of tetanus toxin from *C. tetani*, which is a time-consuming and technically demanding procedure. Several recombinant tetanus toxin derivatives, including nontoxic holotoxoids (16, 25) or subunit domains (16), have also been evaluated as potential new vaccines. Fragment C, the 50-kDa carboxy-terminal portion of tetanus toxin (21, 32), is nontoxic but has ganglioside (20, 22, 31) and protein binding (36) activities. Preparations of recombinant fragment C purified from *Escherichia coli* (28), yeast cells (34), and cultured insect cells (2) will induce protective immune responses against tetanus toxin following parental immunization. In addition, oral vaccines against tetanus toxin based on *Salmonella* spp. as carriers of fragment C are under development (3, 4). We have therefore used fragment C as a model antigen for evaluating DNA-mediated immunization as a system for vaccination against bacterial diseases.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* JM109 (Stratagene, Cambridge, United Kingdom) served as a host for DNA manipulation experiments. All bacterial strains were propagated in Luria broth or on Luria agar (13) supplemented with ampicillin (50 μg/ml) where appropriate. DNA manipulations were carried out as instructed by the manufacturer or as described by Sambrook et al. (35).

Construction of pcDNA3/tetC. The eukaryotic expression vector pcDNA3/tetC, containing the synthetic gene encoding fragment C of tetanus toxin under the control of the human cytomegalovirus major intermediate-early promoter/enhancer region (CMV promoter), was constructed as shown in Fig. 1. *Taq* DNA polymerase (Perkin-Elmer Cetus, Stoke, Staffordshire, United Kingdom) was used in the PCR as instructed by the manufacturer. The synthetic oligonucleotide primers were synthesized in an Applied Biosystems model 392 DNA/RNA synthesizer. Restriction and modification enzymes were purchased from Boehringer Mannheim (Lewes, East Sussex, United Kingdom) and used as recommended by the manufacturer. pcDNA3/tetC was purified by alkaline lysis of chloramphenicol-treated cultures followed by cesium chloride density gradient centrifugation (35). The structure of the purified plasmid was confirmed by separating restriction endonuclease digestion products of the plasmid in agarose gels. Partial DNA sequencing by the dideoxy-chain termination (35) method confirmed that the sequence of the CMV promoter was intact and that an ACC sequence had been successfully inserted immediately upstream of the start codon of the fragment C open reading frame.

Expression of fragment C in vitro. Chinese hamster ovary (CHO) cells were transfected with either pcDNA3/tetC or control DNA, using the calcium phosphate precipitation method described by Sambrook et al. (35). The transfected CHO cells were harvested 72 h later and resuspended in sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) sample buffer. Samples were immediately heated at 100°C for 5 min and cleared by centrifugation in a bench microcentrifuge. Aliquots were analyzed by SDS-PAGE and Western blotting (immunoblotting) (40). For immunoblots, nitrocellulose membranes were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (pH 7.2) (PBS) and then incubated at room temperature for 1 to 2 h with a mouse anti-fragment C antibody diluted in 1% BSA–PBST (PBS plus 0.05% Tween 20). Membranes were incubated with anti-mouse immunoglobulins conjugated to

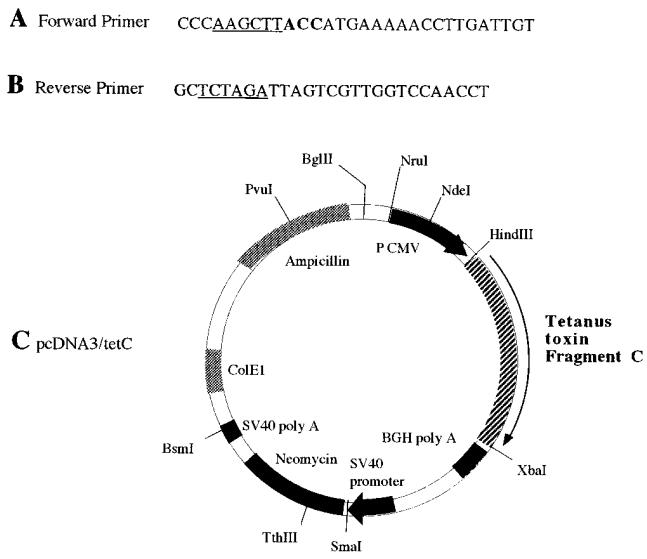

FIG. 1. Construction of pcDNA3/tetC. The synthetic gene encoding tetanus toxin fragment C was amplified from plasmid pTET*nir*15 by PCR. A *Hin*dIII restriction endonuclease site followed by the sequence ACC was incorporated into the forward primer immediately upstream of the start of the gene encoding fragment C (A). The reverse primer (B) contained an *Xba*I restriction endonuclease site immediately downstream of the end of the gene. Initially, the 1,379-bp product of PCR was ligated into the intermediate vector pGEM-T (Promega, Southampton, United Kingdom) to form plasmid pGEM-T/tetC. The gene encoding fragment C was then excised from pGEM-T/tetC by digestion with restriction endonucleases *Xba*I and *Hin*dIII and cloned into pcDNA3 (Invitrogen, R&D Systems, Abingdon, United Kingdom) cleaved with the same enzymes to construct the eukaryotic expression vector pcDNA3/tetC (C). SV40, simian virus 40; BGH, bovine growth hormone.

horseradish peroxidase (Sigma, Poole, Dorset, United Kingdom), and reactive polypeptides were visualized by using 4-chloro-1-naphthol (Sigma) as the substrate. For detecting fragment C in *E. coli*(pcDNA3/tetC), bacterial samples were grown in Luria broth to stationary phase, and 1-ml aliquots were harvested by centrifugation and resuspended in 200 μl of SDS-PAGE sample buffer. These samples were then subjected to SDS-PAGE and Western blotting as described above.

Intramuscular immunization of mice. Six- to eight-week-old female BALB/c mice, bred by B&K Universal Ltd. (Scunthorpe, Humberside, United Kingdom), were used in this study. For DNA immunizations, plasmid DNA was purified by cesium chloride gradient centrifugation, sterilized by ethanol precipitation, and resuspended in PBS at a concentration of 1 mg/ml. DNA concentration was determined from the optical density at 260 nm (OD_{260}) and comparison with DNA standards in ethidium bromide-stained agarose gels. Mice were immunized intramuscularly at three sites in each quadriceps muscle with a total of 100 μg of plasmid DNA, using a 27-gauge needle. For protein immunizations, mice were immunized with 10 μg (unless otherwise stated) of purified fragment C in PBS in the same manner as used for the DNA immunizations.

Measurement of humoral antibody responses by ELISA. Mice were bled from the tail at 1-week intervals following two immunizations with either plasmid DNA or purified fragment C protein. Individual sera were analyzed in an enzyme-linked immunosorbent assay (ELISA) against purified fragment C. In brief, each well of Costar EIA plates was coated overnight at 4°C with 50 μl of a solution of 1 μg of fragment C per ml. After blocking with 1% BSA–PBST, plates were incubated with appropriate dilutions of mouse sera overnight at 4°C. Anti-mouse immunoglobulin–horseradish peroxidase conjugate (Dako, High Wycombe, United Kingdom) was used as the second antibody, and the reaction was visualized with *o*-phenylenediamine dihydrochloride (Sigma). Finally, OD_{490} values were determined by using a Ceres 900HDi ELISA plate reader (Bio-tek Instruments, Inc.). Titer was calculated as the reciprocal of the serum dilution that gave an OD_{490} of 0.3 above that of the preimmune sera.

A similar protocol was followed for ELISA assessing immunoglobulin M (IgM), IgG, and IgG subclass titers against fragment C. Sera from individual mice immunized with pcDNA3 were titrated. Monoclonal antibodies against mouse IgM, IgG, and different IgG subtypes conjugated to biotin (PharMingen, Hull, Humberside, United Kingdom) were used as second antibodies and detected by a further 1-h incubation with streptavidin-horseradish peroxidase conjugate (Sigma).

Splenocyte proliferation assays. Two mice immunized and boosted with fragment C, pcDNA3/tetC, or pcDNA3 were sacrificed on different days after immunization and splenectomized. Single-cell suspensions of each spleen were prepared, and proliferation assays were carried out on washed splenocytes as described previously (39). Different dilutions of fragment C purified from *Pichia pastoris* (6) were used as recall antigens. Controls included cells stimulated with either medium or concanavalin A (Sigma). Cellular proliferation was measured by incorporation of [^{3}H]thymidine during the last 18 h of culture. The stimulation index was defined as the ratio of counts per minute of stimulated and medium-only cultures.

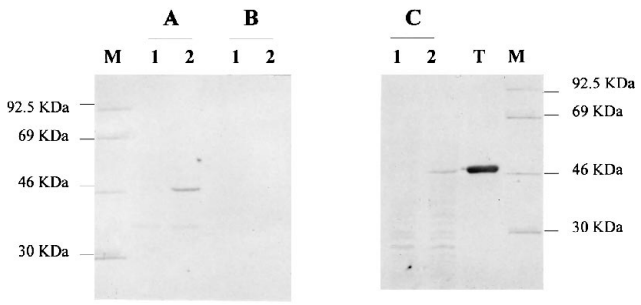

FIG. 2. Expression of fragment C in *E. coli* JM109 and CHO cells. The expression of fragment C by *E. coli* JM109 cells that had been transformed with pcDNA3 (A, lane 1) or pcDNA3/tetC (A, lane 2) was determined by Western blotting as described in Materials and Methods. Similarly, the presence of fragment C in cesium chloride gradient-purified pcDNA3 (B, lane 1) or pcDNA3/tetC (B, lane 2) was analyzed, and the expression of fragment C in CHO cells 72 h following transfection with the same plasmid DNAs was detected (pcDNA3 [C, lane 1]; pcDNA3/tetC [C, lane 2]). One-tenth microgram of purified fragment C protein (T) was run on the gel as a positive control. The molecular weight standards (M) are indicated.

Measurement of cytokines by ELISA. Splenocyte proliferation assays were carried out as described above. Tissue culture supernatants, overlaying the splenocytes stimulated with recall antigen at a concentration of 1 μg/ml or with medium alone, were harvested 72 h following stimulation and stored at −70°C prior to analysis. The concentrations of cytokines gamma interferon (IFN-γ) and interleukin-5 (IL-5) in the tissue culture supernatants were measured by ELISA. Each well of Costar EIA plates was coated overnight at 4°C with 50 μl of a solution of 2 μg of monoclonal antibody against either murine IFN-γ or murine IL-5 (PharMingen) per ml. After blocking for 2 h at room temperature with 10% fetal calf serum (FCS) in PBS, plates were incubated with neat tissue culture supernatants and with known concentrations of the appropriate cytokine diluted in 10% FCS fetal calf serum–PBS. Following overnight incubation at 4°C, the cytokines were detected with monoclonal antibodies against the murine cytokines conjugated to biotin (PharMingen). The ELISAs were developed with the streptavidin-horseradish peroxide system described above, and the concentrations of the cytokines within the supernatants were determined from the standard curve.

Tetanus toxin challenge. Twice-immunized BALB/c mice were each challenged with 50 ng (500 × 50% lethal dose) or 5 ng (50 × 50% lethal dose) of tetanus toxin as previously described (15). Each mouse was injected subcutaneously in the scruff of the neck with tetanus toxin in PBS. Mice that developed symptoms of paralysis were humanely euthanized, and survivors were recorded after 4 days.

RESULTS

Expression of fragment C from pcDNA3/tetC in vitro. Expression of fragment C in both prokaryotic and eukaryotic systems, following transformation or transfection with pcDNA3/tetC, was investigated. A 50-kDa protein band, corresponding to fragment C, was observed following SDS-PAGE and Western blotting of protein extracts prepared from overnight cultures of *E. coli*(pcDNA3/tetC) (Fig. 2A). A similar band was not observed in protein extracts prepared from *E. coli* harboring pcDNA3; therefore, pcDNA3/tetC appeared to direct the expression of low levels of fragment C in *E. coli*. Fragment C protein could not be detected in cesium chloride gradient-purified pcDNA3/tetC samples (Fig. 2B). pcDNA3/tetC was transfected into CHO cells, and 72 h later, protein extracts were analyzed by SDS-PAGE and Western blotting. A polypeptide corresponding to fragment C was visible in protein

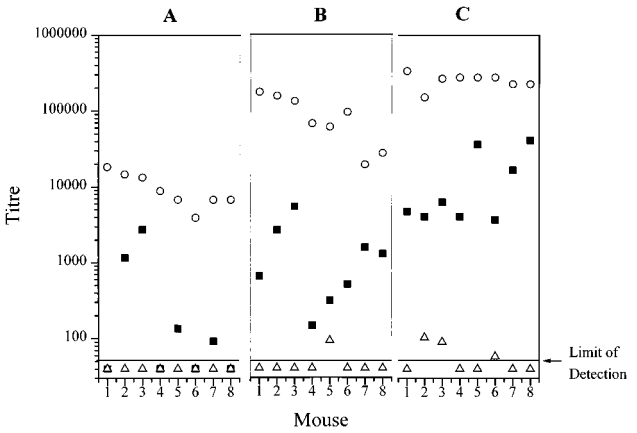

FIG. 3. Serum response against pcDNA3/tetC. The levels of total immunoglobulin against fragment C were determined in individual mice 17 days (A) following intramuscular immunization with a single dose of 100 μg of pcDNA3/tetC (■), 100 μg of pcDNA3(△), or 10 μg of purified Fragment C (○). Mice were boosted with the same immunogens, and the total anti-fragment C immunoglobulins in sera taken from each mouse were determined 7 (B) and 21 (C) days after boosting. Titer was calculated as the reciprocal of the serum dilution that gave an OD_{490} of 0.3 above that of the preimmune sera. The detection limit of the assay was a titer of 50.

extracts prepared from CHO cells transfected with pcDNA3/tetC but not those transfected with pcDNA3 (Fig. 2C). Thus, pcDNA3/tetC appeared to direct the expression of fragment C in eukaryotic cells in vitro.

Humoral responses in BALB/c mice following immunization with pcDNA3/tetC. Mice were immunized intramuscularly with either pcDNA3/tetC or purified fragment C protein, and humoral immune responses were analyzed over the following weeks. Two weeks following the first immunization with 100 μg of pcDNA3/tetC, anti-fragment C immunoglobulins were observed in the sera of 4 of 10 mice (Fig. 3A). A second immunization was sufficient to induce production of anti-fragment C antibodies in all of the mice immunized with this plasmid (Fig. 3B), with peak titers (geometric mean = $\log_{10}$ 4.06 ± 0.43) being observed 21 days later (Fig. 3C). However, during a 5-week period following the second immunization, antibody titers did not exceed the peak titers induced by intramuscular immunization with 10 μg of purified fragment C (geometric mean = $\log_{10}$ 5.52 ± 0.06). The anti-fragment C immunoglobulin isotypes produced in the serum of the DNA- or protein-immunized mice were very similar, with IgG dominating the antibody response 21 days following the second immunization (results not shown). However, the IgG subclass profiles of the DNA- and protein-immunized mice were distinctly different (Fig. 4). The major IgG subclass induced by DNA immunization was IgG2a, whereas intramuscular immunization with purified protein induced the production of IgG1.

Splenocyte proliferative responses following immunization with pcDNA3/tetC. Proliferative responses to fragment C in splenocytes of BALB/c mice were measured 14 and 21 days following immunization with either DNA or protein. Anti-fragment C proliferation was observed in splenocytes taken from mice that had been immunized with pcDNA3/tetC but not in splenocytes taken from mice immunized with pcDNA3. Similar levels of anti-fragment C proliferation occurred in the splenocyte cultures that had been prepared from mice immunized with purified fragment C protein (Fig. 5).

Cytokine secretion by splenocytes following immunization with pcDNA3/tetC. Cytokines secreted by fragment C-stimulated splenocytes from mice immunized with pcDNA3/tetC,

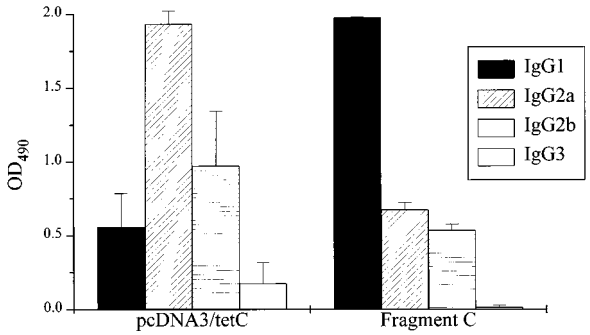

FIG. 4. IgG subclasses against fragment C induced by immunization with pcDNA3/tetC or purified fragment C. Mice were immunized intramuscularly with 100 μg of pcDNA3/tetC or 10 μg of fragment C and boosted 2 weeks later in an identical manner. Anti-fragment C IgG subclasses were detected by ELISA in sera taken from individual mice 21 days following two immunizations with either pcDNA3/tetC or fragment C. Each column represents the mean OD_{490} measured at a serum dilution of 1/250 ± 1 standard deviation.

pcDNA3, or purified fragment C protein or unimmunized mice were compared (Table 1). IFN-γ was produced by splenocytes from mice immunized with pcDNA3/tetC but not with pcDNA3 or PBS. In contrast, IL-5 secretion was detected in the culture media of spleen cells from mice immunized with purified fragment C protein but not with pcDNA3/tetC, pcDNA3, or PBS.

Protection of mice against challenge with tetanus toxin following immunization with pcDNA3/tetC. Following two intramuscular immunizations with pcDNA3/tetC, pcDNA3, or fragment C protein, BALB/c mice were challenged with a lethal dose of tetanus toxin. Initially, mice immunized with pcDNA3/tetC were fully protected against lethal challenge with tetanus toxin (Table 2). In a subsequent experiment, however, 70% of the mice were protected against challenge. In both experiments, mice immunized with purified fragment C protein were completely protected against challenge, while those immunized with pcDNA3 or PBS died within 48 h of the challenge.

DISCUSSION

The eukaryotic expression vector pcDNA3/tetC, encoding a partially synthetic tetanus toxin fragment C gene, was constructed and used to assess the potential of DNA-mediated immunization against tetanus. pcDNA3/tetC was shown ini-

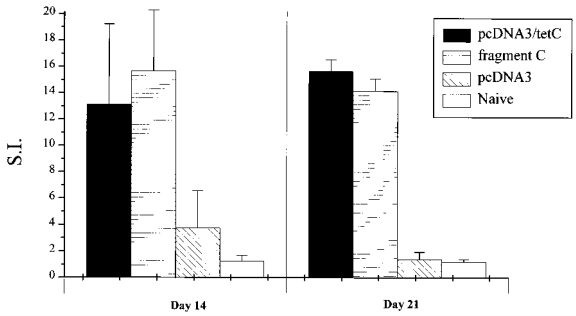

FIG. 5. Proliferative responses in splenocytes induced by immunization with pcDNA3/tetC or fragment C. Mice were immunized twice with pcDNA3/tetC, pcDNA3, or fragment C. Fourteen and 21 days following the second immunization, proliferative responses to fragment C were determined in splenocytes isolated from two mice per group. As a negative control, proliferation to fragment C in splenocytes prepared from naive mice was also measured. Each column represents the mean stimulation index (S.I.) ± 1 standard error.

TABLE 1. Production of cytokines IFN-γ and IL-5 following immunization with pcDNA3/tetC or fragment C

Vaccine	Fragment C[a]	IFN-γ (U/ml)[b]	IL-5 (ng/ml)[b]
pcDNA3/tetC	+	29.8	<0.1
	−	<10	<0.1
pcDNA3	+	<10	<0.1
	−	<10	<0.1
Fragment C	+	<10	0.47
	−	<10	<0.1
None, naive mice	+	<10	<0.1
	−	<10	<0.1

[a] Splenocytes were prepared and stimulated with 1 μg of purified fragment C 21 days following the second immunization as described in the text.
[b] Mean concentration of cytokine determined by ELISA in three supernatant samples.

TABLE 2. Protection of BALB/c mice against challenge with tetanus toxin following immunization with pcDNA3/tetC

Group	Vaccine	Dose[a] μg	No. of mice surviving tetanus toxin challenge
1[b]	pcDNA3/tetC	100	6
	Fragment C	10	6
	pcDNA3	100	0
	Naive		0
2[c]	pcDNA3/tetC	100	7
	Fragment C	1	10
	pcDNA3	100	0
	Naive		0

[a] BALB/c mice were twice immunized intramuscularly with the indicated dose as described in the text.
[b] Six mice were challenged with 50 ng of tetanus toxin per mouse (500 × 50% lethal dose).
[c] Ten mice were challenged with 5 ng of tetanus toxin per mouse (50 × 50% lethal dose).

tially to direct expression of fragment C in CHO cells in vitro. BALB/c mice immunized with purified preparations of pcDNA3/tetC DNA, which contains a CMV-derived promoter, mounted significant humoral and cellular immune responses to fragment C. Plasmid DNAs encoding reporter genes under control of the CMV promoter have been shown to induce high levels of expression in a wide range of mammalian tissues (5). Moreover, this promoter system has previously been used to induce an immune response to a wide range of antigens in vivo following intramuscular immunization (8, 12, 24, 27, 29, 37, 41, 48). In contrast to the native gene encoding fragment C, the partially synthetic gene had previously been shown to direct the expression of high levels of fragment C in *E. coli* (28) and *Saccharomyces cerevisiae* (34). Therefore, the use of a codon-optimized gene may have contributed to the efficient transcription of fragment C in higher eukaryotic cells.

A humoral response against fragment C was induced in BALB/c mice following two intramuscular immunizations with pcDNA3/tetC. Similar humoral responses have been observed in several other systems employing direct immunization of plasmid DNA. For example, low antibody titers (10^1 to 10^2) were observed against *Plasmodium yoelii* circumsporozoite protein in mice following a single DNA-mediated immunization; however, titers were increased to levels of 10^4 to 10^5 following a second DNA immunization (37). Xiang et al. have observed similar responses to a DNA immunization system encoding the rabies virus glycoprotein (46). In contrast, significant antibody titers were observed following a single DNA-mediated immunization with influenza virus A nucleoprotein (41) or hepatitis B virus surface antigen (11). However, no antibody responses were observed in the latter study until 4 weeks after immunization. Since plasmid DNAs encoding expressed reporter genes have been shown to persist in mouse muscle for over 1 year (44), a higher humoral response against pcDNA3/tetC might have been obtained after a single dose if antibody levels were monitored for a longer time period prior to boosting. Although DNA immunization with fragment C did not induce antibody titers that were higher than those observed following immunization with purified fragment C protein, DNA-mediated immunization with fragment C induced sufficient antibody titers in most mice to protect against lethal challenge with tetanus toxin. Inconsistent delivery of the plasmid DNA to muscle cells may have resulted the failure to observe complete protection of the DNA-immunized mice. Several methods for improving plasmid DNA delivery and uptake by muscle cells which could be investigated to enhance the immune response to fragment C have been described (10, 45). In addition, gene gun delivery of plasmid DNA to the epidermis, which has been shown to elicit an immune response with considerably less DNA than is required for intramuscular immunization (19), could be used to deliver pcDNA3/tetC in future experiments.

Proliferative responses in splenocytes stimulated with fragment C in cell culture indicated that DNA or protein immunization induced similar levels of splenocyte proliferation against fragment C. However, stimulated splenocytes from DNA-immunized mice secreted significant amounts of IFN-γ, a Th1-associated cytokine, whereas splenocytes from mice immunized with purified protein secreted IL-5 in the absence of IFN-γ, a Th2-type cytokine profile. These findings were supported by the observation that DNA-mediated immunization predominantly induced IgG2a production, which is normally IFN-γ dependent. In contrast, the major IgG subclass induced by immunization with the purified fragment C protein was IgG1. Th1-type T-helper cell proliferation has previously been observed after DNA-mediated immunization with rabies virus glycoprotein (46, 47) and *Leishmania major* glycoprotein gp63 (48) and after multiple immunization with *P. yoelii* circumsporite protein (30). Thus, DNA-mediated immunization has now been shown to induce a Th1 T-helper response against several antigens of very different origin. The induction of a Th1-type response by DNA immunization may therefore be related to this method of antigen delivery rather than the properties of the individual antigen.

Immunity to tetanus is dependent on a humoral immune response against tetanus toxin. However, the induction of IFN-γ indicates that DNA-mediated immunization with fragment C probably induced a Th-1 type T-helper response, which is usually associated with cell-mediated immunity. If Th1-type responses are widely observed following DNA-mediated immunization with bacterial antigens, this vaccination system may be effective against intracellular bacterial pathogens, such as salmonellae or mycobacteria. However, recent work in which a gene gun was used to deliver plasmid DNA encoding human immunodeficiency virus type 1 glycoprotein 120 epidermally has shown that a Th2-type response was induced after multiple immunization (17). Thus, similar systems could possibly be exploited to induce Th2 responses against bacteria antigens. Manipulation of the T-helper response may also be possible by coimmunization with plasmids expressing cytokines that promote either Th1- or Th2-type responses (33).

DNA-mediated immunization clearly has the potential to induce biologically relevant immune responses against bacte-

rial antigens. This system could therefore be exploited as a means of vaccination against bacterial disease. Although safety considerations may limit the use of such vaccines in humans, nucleic acid vaccination against bacteria diseases could be widely used in animals. Certainly antitetanus immunity is likely to be considered as a component of any future DNA-based immunization program. DNA-mediated immunization with bacterial antigens also has several potential routine uses. This system may provide an interesting method for the production of antisera against potential bacterial antigens, eliminating the necessity to obtain purified preparation of the antigens. Furthermore, if manipulation of T-helper responses is possible, DNA-mediated immunization will provide a useful tool for determining the nature of the immune response against pathogenic bacteria.

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