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# DNA immunisation against the CFA/I fimbriae of enterotoxigenic *Escherichia coli* (ETEC)

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**Abstract**

The CFA/I fimbria promotes the attachment of enterotoxigenic *Escherichia coli* (ETEC) to the surface of human enterocytes. The generation of a protective immune response requires the induction of antibodies able to block the CFA/I-mediated binding of ETEC to receptors located on the small intestine epithelium or on the surface of human red blood cells, in hemagglutination tests. An eukaryotic expression plasmid, pBLCFA, encoding the CFA/I gene under the control of the human cytomegalovirus major immediate-early promoter was constructed as a prototype DNA vaccine against ETEC. pBLCFA-tranfected BHK-21 cells secreted a peptide cross-reacting with a monoclonal antibody raised against CFA/I subunits. BALB/c mice immunized intramuscularly with one or two doses of purified pBLCFA developed CFA/I-specific serum antibodies for at least 52 weeks, composed predominantly of the IgG1 subclass. pBLCFA-induced antibodies bind mainly to epitopes exposed on the surface of intact CFA/I fimbriae and do not react with immune recessive epitopes found in other ETEC fimbra sharing amino acid homologies with CFA/I. Furthermore, pBLCFA-induced antibodies were able to block the adhesive properties of the CFA/I fimbriae, as evaluated by the ability to inhibit the hemagglutination promoted by CFA/I-expressing ETEC cells. These results suggest that secretion of CFA/I encoded by pBLCFA preserves important conformational epitopes required for the generation of protective antibodies against the adhesive properties of the CFA/I fimbriae and open new perspectives for the development of DNA vaccines against enteric bacterial pathogens. © 2000 Published by Elsevier Science Ltd.

*Keywords:* DNA vaccines; ETEC; CFA/I fimbriae; Diarrheal disease

## 1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute diarrhea in children in developing countries and in travelers who visit those areas [1]. In fact, ETEC has recently been recognized by the World Health Organization (WHO) as a priority target to be controlled by vaccination [2]. The two most important events in ETEC pathogenesis are adherence of bacteria to the small intestine epithelial surface, a host-specific process mediated by colonization factor antigens (CFs or CFAs), and delivery of enterotoxins. Some experimental approaches, such as the ability to hemagglutinate group A human red blood cells or attachment to Caco-2 cells, have been designed to evaluate the binding properties of different ETEC strains [3]. CFA/I was the first CF described in ETEC and CFA/I-expressing strains are widely distributed in endemic areas as South America and Asia [4–6]. It is a fimbrial adhesin composed of a single 15 kDa subunit endowed with structural and functional (adhesion) properties [4–6]. Significant amino acid homology has been found at the N-terminal region of different ETEC fimbra, such as CFA/I, CS1, CS2, CS4 and PCFO166, but antibodies raised against conformational epitopes of intact CFA/I are usually specific to strains expressing this fimbriae [7,8].

Oral administration of purified CFA/I fimbriae can confer partial protection against homologous strains,

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but require large amounts of protein due to its lability in the gastric and intestinal environment [9]. The ETEC vaccine presently available for humans relies on the oral administration of formalin-killed strains expressing CFA/I, CFA/II and CFA/IV, plus the purified B-subunit of the heat labile toxin [10]. Although safe and simple to use, the oral LT-B subunit whole-cell ETEC vaccine requires repeated immunizations and induces short-term protection, probably due to the reduced immunogenicity of non replicating vaccine formulations. Therefore, new and efficient vaccine strategies against ETEC are clearly required.

DNA vaccines have provided a promising approach to vaccination against various pathogens leading to the induction of long lasting immune responses [11–14]. A proposed advantage of DNA vaccines relies on the fact that in situ production of antigens by transfected host cells would retain a more natural conformation in regard to those produced in bacterial cells and used as subunit vaccines [15]. However, antigens encoded by DNA vaccine vectors may frequently lead to inadequate antibody responses when the encoded antigens are derived from extracellular replicating bacteria. For example, bacterial antigens encoded by DNA vaccines may loose important conformational epitopes required for the generation of neutralizing antibodies [16] or for attaining elevated systemic levels when the antigens are accumulated inside the transfected host cells [17].

We have previously described the construction of an eukaryotic expression vector (pRECFA) encoding a recombinant CFA/I protein fused to N- and C-terminal sequences of the glycoprotein D from herpes simplex virus type 1 (HSV gD1) and targeted to the cytoplasmic membrane of transfected mammalian cells [16]. Intramuscular (i.m.) inoculation of purified pRECFA in BALB/c mice elicited strong and long-term systemic antibody responses. However, the adhesive properties of intact CFA/I fimbriae were not inhibited by pRECFA-induced CFA/I-specific antibodies, suggesting that important conformational epitopes were lost in regard to the antigen derived from bacterial cells [16,18].

In an attempt to induce an antibody response able to inhibit the adhesive properties of the CFA/I fimbriae, in the present work we cloned the gene encoding the CFA/I (*cfaB*) in an eukaryotic expression vector (pBLCFA), which drives the secretion of the CFA/I protein under the control of the human cytomelagovirus (CMV) immediate-early promoter. In contrast to pRECFA, mice i.m. immunized with pBLCFA produced CFA/I-specific antibodies which neutralize the adhesive properties of the CFA/I fimbriae. These results indicate that secretion of the antigen encoded by a DNA vaccine has drastic effects on the properties of the antibody response.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The ETEC strain 4011-1 (CFA/I, O153:H45, ST) [19] was used to clone the *cfaB* gene. The ETEC strain 258909-3 (CFA/I, O128:H?, ST/LT) [20] and the corresponding isogenic CFA/I-negative strain, 258909-3M [7], were used in inhibition enzyme-linked immunosorbent assay (ELISA), agglutination and inhibition of hemagglutination tests. Purified CFs, kindly provided by Dr A.M. Svennerholm (Gotheborg University, Sweden), were isolated from strains H10407 (CFA/I, O78:H11, ST/LT) [8], 60R936 (CS1, O139:H28) [21] and E11881A (CS4, CS6, O25:H42, ST/LT) [22]. Unless otherwise stated cultures were prepared in Luria broth. For inhibition ELISA, agglutination and inhibition of hemagglutination (IHA) tests the 258909-3 and 258909-3M strains were grown overnight on Casamino acids–yeast extract agar plates [3] at 37°C.

### 2.2. Plasmid construction

The *cfaB* gene, without promoter and signal sequences, was amplified by PCR and cloned in pkCMVintBL, kindly provided by Dr R. Zaug (Vical Inc., San Diego, CA). pkCMVintBL is a modified pUC18-based vector which contains the human cytomegalovirus (CMV) immediate-early promoter and intron A, the human tissue plasminogen activator (TPA) signal sequence and the SV40 early polyadenylation signal [23,24]. Amplification of the *cfaB* gene was carried out with template DNA from the ETEC strain 4011-1 and two synthetic oligonucleotide primers (sense 5′–GGG GGG ATC CGT GAG TGC TTC AGC A and antisense 5′–GGG GAG ATC TGG ACC CCA AAG TCA T) which contain restriction sites for *Bam*HI and *Bgl*II, respectively. The reactions were performed in a Gene ATAQ controller (Pharmacia LKB, Uppsala, Sweden) programmed as follows: 80°C for ten min, 35 cycles of 92°C for two min, 55°C for two min and 72°C for five min, and an extension step at 72°C for ten min at the end of the cycle. The amplified products were electrophoresed on a 1% agarose gel, recovered with glass beads (Geneclean), restricted with *Bam*HI and *Bgl*II, and ligated to pkCMVintBL previously digested with *Bam*HI/*Bgl*II. Recombinants were screened by restriction mapping and sequencing by the dideoxy chain termination method [25]. The recombinant plasmid, pBLCFA, targets secretion of the encoded protein to the extracellular space, mediated by the TPA signal sequence. Large-scale purification of pBLCFA and pkCMVintBL were conducted by equilibrium density cesium chloride (CsCl) gradient centrifugation, repeated twice, followed by sterilization by ethanol precipitation, suspension in sterile phosphate-buffered saline (PBS)

and stored at −20°C until use. DNA concentration was determined at $A_{260nm}$ and visual inspection of ethidium bromide-stained agarose gels using DNA fragments of known concentrations. All DNA manipulations followed standard procedures [26] or instructions supplied by the manufacturers.

### 2.3. In vitro expression of CFA/I by bacterial and mammalian cells

BHK-21 cells were transfected with either pkCMV-intBL or pBLCFA with lipofectamine (Gibco–BRL, Life Laboratories, Bethesda, USA) in Optimen medium (Gibco–BRL) under conditions suggested by the manufacturer. The cells and culture supernatants were harvested 24 and 48 h following transfection. Whole-cell extracts and culture supernatants, precipitated with 10% TCA, were suspended in SDS–PAGE sample buffer [26], boiled for five min and proteins sorted in 15% polyacrylamide gels followed by Western blotting. Nitrocellulose membranes were blocked overnight with 1% w/v bovine serum albumin (BSA) in PBS (pH 7.2) at 4°C, followed by one h incubation at 37°C with MAb 84, a CFA/I subunit-specific mouse monoclonal antibody [27]. Membranes were washed in 0.05% v/v Tween 20–PBS (PBST) and incubated with rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Sigma, St. Louis, MO) for one h at 37°C. Membranes with bound antibodies were developed with the ECL kit (Amersham, Buckinghamshire, UK) and exposed to Kodak X-Omat films for one–two min.

### 2.4. Immunization procedure

Four- to six-week-old male BALB/c mice, purchased from Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil), were injected in the tibialis anterior muscle of each hind limb with 50 μg of plasmid DNA dissolved in 50 μl of PBS (100 μg/dose), using 27-gauge needles. Two independent experiments were performed with two groups ranging from six to nine animals. The first group was immunized with a single pBLCFA dose while the second group was inoculated with two doses of pBLCFA given two weeks apart. Control groups formed by six animals were immunized with two doses of pkCMVintBL. Mice were bled, by retro-orbital puncture, before inoculation (preimmune sera) and at various time intervals following the immunizations. Harvested sera were stored at −20°C until testing.

### 2.5. Detection of CFA/I-specific antibodies by ELISA

CFA/I-specific ELISA was performed with serum samples harvested from each mouse using purified CFA/I fimbriae as solid-phase bound antigen. Wells of MaxiSorp plates (Nunc, Roskilde, Denmark) were coated at 37°C for one h with 0.1 μg of CFA/I subunits suspended in PBS. After overnight blocking at 4°C with 2% w/v skim milk in PBST and washing with PBST, plates were incubated with serially diluted sera and kept for one h at 37°C. For detection of IgG, IgG1 and IgG2a, rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase (Southern Biotechnologies Associates, Birmingham, USA) were used as the second antibody for one h at 37°C. Both secondary antibodies used to determine IgG subclasses were highly specific and had quantitatively similar reactivity in ELISA, thus allowing direct comparison between the levels of each subclass. The anti-CFA/I fimbriae rabbit serum, kindly provided by Dr David Maneval (Center for Vaccine Development, Baltimore, USA), was raised after parental immunization with intact CFA/I fimbriae. Detection was carried out with goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) used as the secondary antibody in ELISA. Reactions were followed in a model 450 microplate reader (Bio-Rad, Rockville, USA) at $A_{490nm}$ using *o*-phenylenediamine dihydrochloride (Sigma) and $H_2O_2$ as enzyme substrate, and 9N $H_2SO_4$ as stopping solution. Titres were established as the reciprocal of the serum dilution that gave an absorbancy above that of the respective preimmune sera. Standard deviations of duplicates or triplicates were always below 5% of the mean.

### 2.6. Inhibition ELISA

Inhibition ELISA was carried out with ETEC cells expressing CFA/I fimbriae (strain 258909-3), as the competitor antigen. Briefly, bacteria were suspended to a cell density of approximately $10^{11}$ colony forming units (cfu) $ml^{-1}$ in PBS and aliquots were mixed with the same volume of the tested sera. After ten min at room temperature, duplicate samples of the mixture were titered by five-fold serial dilutions using ELISA plates covered with intact CFA/I fimbriae. The same experimental procedure was carried out with the isogenic CFA/I-negative ETEC strain 258909-3M, grown under the same conditions, as a negative control. The rabbit anti-CFA/I serum was employed as a positive control able to bind epitopes exposed on the surface of intact fimbriae. MAb 84 reacts with a linear epitope hidden on the structure of the CFA/I fimbriae [27].

### 2.7. Reactivity of antibodies to different ETEC fimbriae

Samples of purified fimbriae (CFA/I, CS1 and CS4) were boiled in SDS–PAGE sample buffer [26] for five min and applied to 15% w/v acrylamide gels. Gels and Western blots were performed as described above, except for the use of serum samples collected from mice immunized with the pBLCFA.

### 2.8. Agglutination assays

The CFA/I-expressing ETEC strain 258909-3 and its isogenic CFA/I-negative derivative, strain 258909-3M, were harvested and suspended in PBS. Samples of the bacterial suspensions (approximately $10^{10}$ cells $ml^{-1}$) were mixed with the same volume of PBS or test sera on a glass slide. The slides were kept in humid chambers for 20 min at room temperature and agglutination of bacterial cells was evaluated by visual inspection. Sera harvested from mice immunized with pkCMVintBL were used as negative controls.

### 2.9. Inhibition of hemagglutination assay (IHA)

Sera diluted in PBS containing 0.5% D-mannose (20 μl) were preincubated on glass slides with the ETEC strains 258909-3 or 258909-3M (O.D. of 0.3 at 600 nm) at room temperature for ten min. The same volume of a 1% suspension of washed human group A erythrocytes diluted in PBS containing 0.5% w/v D-mannose was added and the slides were incubated at room temperature for 20 min in humid chambers. The hemagglutination results were evaluated by visual inspection. Serum pools of mice immunized with pkCMVintBL were used as negative controls.

## 3. Results

### 3.1. pBLCFA-transfected BHK-21 cells secrete CFA/I into the growth medium

As shown in Fig. 1, a single peptide cross-reacting with MAb 84 was detected in whole-cell extracts and culture supernatants of pBLCFA-transfected BHK-21 cells but not in cultures transfected with pkCMVintBL. No peptide cross-reacting with the CFA/I-specific MAb84 was found in protein extracts of bacterial cells transformed with pBLCFA or in aliquots of purified plasmid DNA preparations (Fig. 1). These results indicate that pBLCFA promotes expression of CFA/I only in eukaryotic cells and drives the secretion of the antigen to the surrounding medium. The amount of CFA/I produced by pBLCFA-transfected BHK-21 cells, both in whole-cell extracts and culture supernatants, was higher in cells incubated for 24 h after transfection than in those incubated for 48 h (Fig. 1), suggesting that, at least in vitro, expression of plasmis-encoded CFA/I is toxic to the host cells.

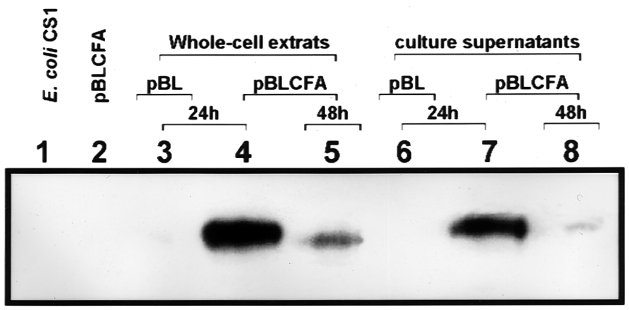


Fig. 1. pBLCFA-driven CFA/I production. Detection of peptides cross-reacting with CFA/I was carried out in Western blots with MAb 84. Lane 1, *E. coli* CS1 transformed with pBLCFA. Lane 2, 50 μg of purified pBLCFA. Whole-cell extracts (lanes 3–5) and culture supernatants (lanes 6–8) were harvested from BHK-21 cells transfected with pkCMVintBL (lanes 3 and 6) or pBLCFA (lanes 4, 5, 7 and 8). Transfected BHK-21 cells were incubated during 24 h (lanes 3, 4, 6 and 7) or 48 h (lanes 5 and 8) before harvesting for protein sorting by SDS–PAGE.

### 3.2. Serum antibody responses in mice immunized with pBLCFA

Only two out of six BALB/c mice inoculated with a single 100 μg dose of pBLCFA developed low anti-CFA/I serum antibody levels four weeks following the immunization (Fig. 2). Serum samples harvested 52 weeks after the initial immunization showed that pBLCFA induced long lasting antibody responses against CFA/I but no significant increase in the CFA/I antibody levels were observed. On the other hand, seven of nine mice inoculated with two 100 μg doses of pBLCFA developed serum anti-CFA/I antibody responses two weeks following the boosting dose (Fig. 2). All mice inoculated with two pBLCFA doses showed positive CFA/I-specific serum antibody responses 52 weeks following the initial dose but, on average, the antibody level detected did not differ significantly from those observed at earlier periods. A broad range of CFA/I-specific antibody titres were observed in serum samples collected at four or 52 weeks following immunization with two doses of pBLCFA (levels ranging from $5 \times 10^2$ to approximately 10u). Determination of serum IgG subclass responses showed that IgG1 was the predominant CFA/I-specific subclass in pBLCFA-vaccinated mice, regardless of the total IgG level, although significant CFA/I-specific IgG2a levels were also detected (Fig. 3).

### 3.3. Epitope specificity of antibodies induced by pBLCFA

Sera from mice immunized with pBLCFA were submitted to inhibition ELISA carried out with strains 258909-3 and 258909-3M. As shown in Fig. 4, following adsorption with CFA/I-expressing ETEC cells, pBLCFA-induced antibodies had significantly reduced reactivity to solid phase bound CFA/I in ELISA, suggesting that surface-exposed epitopes were predominantly recognized. Anti-CFA/I antibodies raised in rabbits immunized with purified CFA/I fimbriae and

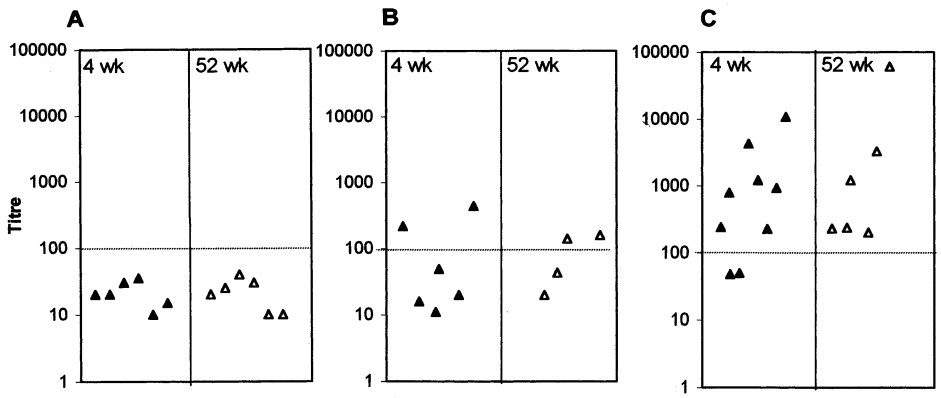


Fig. 2. CFA/I-specific serum IgG responses in BALB/c mice immunized with pBLCFA or pkCMVintBL. Mice were immunized with two 100 μg doses of pkCMVintBL (A), one (B) or two (C) 100 μg doses of pBLCFA. Serum samples were harvested four weeks (closed symbols) and 52 weeks (open symbols) following the initial immunization and evaluated by CFA/I-specific IgG-ELISA. Some mice immunized either with one or two DNA doses died during the course of this study due to undetermined causes. Cut-off values for positive reactions were indicated by the dashed line.

binding exclusively to surface-exposed conformational epitopes, showed a similar reaction pattern in inhibition ELISA (Fig. 4). In contrast, MAb 84, which recognizes a linear epitope hidden in a non-exposed domain of the fimbriae, did not react with live ETEC cells expressing intact CFA/I fimbriae, as evaluated in inhibition ELISA experiments. As expected, sera from mice immunized with pBLCFA did not cross-react with dissociated CS1, PCFO166, or CS4, ETEC fimbriae which share significant amino acid homology with CFA/I mainly at non-exposed domains, (data not shown). Taken together, these results indicate that CFA/I-specific antibodies elicited in mice immunized with the pBLCFA predominantly recognize surface-exposed epitopes found only on intact CFA/I fimbriae.

### *3.4. pBLCFA-induced antibodies inhibit the adhesive properties of the CFA/I fimbriae*

The ability to block the hemagglutination promoted by CFA/I-expressing ETEC cells was used as an approach to investigate the anti-adhesive properties of antibodies elicited by pBLCFA. Sera from mice immunized with two doses of pBLCFA were able to inhibit the hemagglutination properties of CFA/I-expressing ETEC cells (Table 1). Antibodies raised in mice immunized with pBLCFA, were also able to agglutinate CFA/I-expressing ETEC cells (Table 1). In contrast, sera harvested from mice immunized with pkCMVintBL were negative for both agglutination and IHA.

## 4. Discussion

In this report we analyzed the antibody responses raised in mice inoculated with a DNA vaccine, pBLCFA, which can drive expression of the CFA/I subunit in eukaryotic but not in bacterial cells. Western blots of in vitro transfected BHK-21 cells showed that CFA/I encoded by pBLCFA was secreted into the surrounding growth medium, suggesting that in vivo pBLCFA-transfected host cells behave in a similar way.

Generation of significant CFA/I-specific IgG responses in mice vaccinated with pBLCFA required two DNA doses although considerable individual variation was observed. The broad range of CFA/I-specific antibody titres may be attributed to the variable in vivo transfection efficiency which could affect the antigen yield and, consequently, activation of B-cell dependent antibody responses. Similar results were reported with other DNA vaccine models [28–30] and some proposed approaches to attain a more uniform antibody response include additional inoculations, co-administration of substances which could increase transfection rates and gene gun delivery [31,32]. Attempts to quantitatively improve the antibody responses elicited in mice vaccinated with pBLCFA are presently under investigation.

In contrast to other DNA vaccines which preferentially induce IgG2a subclass responses [12,28,32–34], the i.m. inoculation of pBLCFA induced a predominant serum IgG1 production, indicative of a Th2-type T-helper response. The continuous production of secreted CFA/I subunits by transfected cells probably represents a determinant factor of a The2-type immune response, since another DNA vector directing the expression of the CFA/I protein to the surface of the cytoplasmic membrane of mammalian cells (pRECFA) preferentially induced a Th1-type immune response [35,36]. On the other hand, secretion of the encoded antigen does not seem to be an essential requirement for long term antibody responses since mice immunized with either or both vectors (pRECFA or pBLCFA) were able to sustain high CFA/I-specific antibody levels for at least 52 weeks [36].

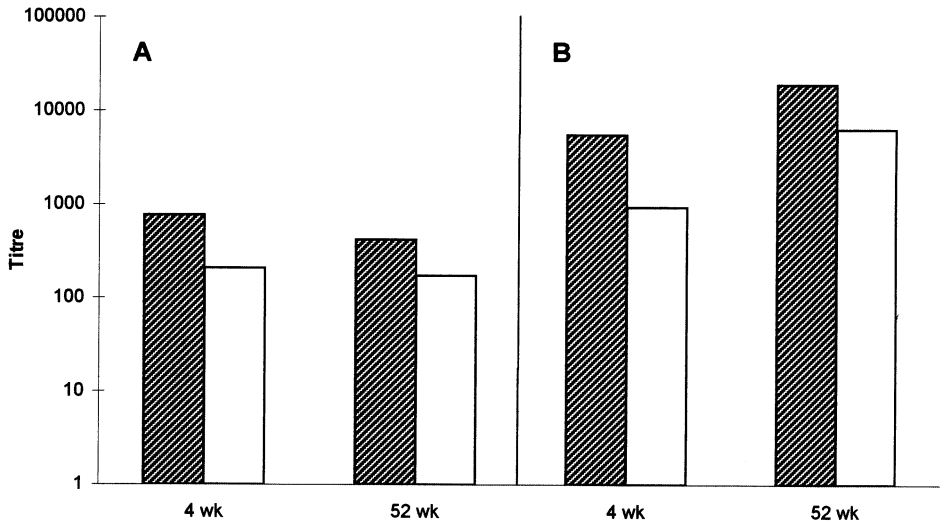


Fig. 3. CFA/I-specific IgG subclass responses in mice immunized with pBLCFA. Sera from mice inoculated with two 100 μg doses of pBLFCA presenting low (A) or high (B) CFA/I-specific antibodies, harvested four or 52 weeks following the initial immunization, were submitted to IgG1 and IgG2a ELISA. Standard deviations of duplicates or triplicates were always below 5% of the means.

Besides differences in the predominant IgG subclasses elicited by immunization with these two plasmids, secretion of the antigen encoded by a DNA vaccine also seems to affect epitope specificity of the induced antibodies. pBLCFA-induced antibodies reacted with surface-exposed epitopes found on native CFA/I fimbriae while antibodies raised against denatured fimbrial subunits or DNA vaccines encoding a membrane-bound CFA/I bound preferentially to linear epitopes hidden on the tertiary structure of the native fimbriae [16,18,37,38]. More relevantly, pBLCFA-induced antibodies which recognize important epitopes of the CFA/I protein and neutralize the adhesive properties of the fimbriae expressed in the bacterial cell, as demonstrated by hemagglutination tests performed with CFA/I-expressing ETEC cells, a feature not observed in mice immunized with DNA vaccines encoding non-secreted form of the CFA/I antigen [16,18]. Such unique aspects of the immune response elicited in mice vaccinated with pBLCFA are probably attributed to the conformation of the encoded antigen which preserve at least some of the surface-exposed epitopes found on intact fimbriae produced by ETEC cells. Moreover, secretion probably increases the availability of CFA/I to macrophages and other antigen-presenting cells (APC), thus favoring the induction of an effective antibody response.

Recently, the usefulness of purified DNA as a vaccine approach against bacteria which replicates extracellularly has been questioned [17]. In the case of the tetanus toxoid [17], subunit vaccines based on the administration of purified native or recombinant fragment C peptide seem to induce stronger and longer protective antibody responses in regard to the same antigen encoded by a DNA vaccine. On the other hand, our results show that the antibody responses against non-invasive bacterial pathogens can be significantly improved through the use of plasmid vectors which promote secretion of the encoded antigens. Such improved antibody responses might reflect either a higher concentration of circulating antigen or a more efficient antigen presentation supporting a better activation of B lymphocytes. The present evidence demonstrated that the design of a DNA vaccine, in regard to the encoded antigen, represents a key step in the development of vaccines against non-invasive bacterial pathogens.

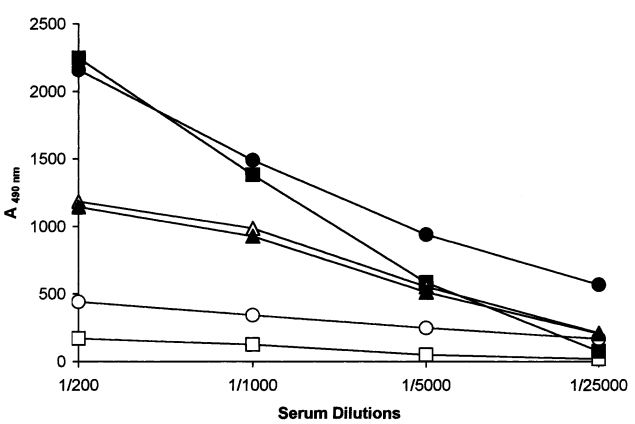


Fig. 4. CFA/I epitope specificity of antibodies raised in mice immunized with pBLCFA in inhibition ELISA. Experiments were carried out with sera harvested from mice immunized with pBLCFA (squares), rabbit serum raised against intact CFA/I fimbriae (circles), or MAb 84 (triangles). Sera were absorbed with live cells of strains 258909-3 CFA/I$^+$ (open symbols) and its isogenic CFA/I-negative derivative, 258909-3M (closed symbols) before analysis by IgG ELISA with purified intact CFA/I fimbriae (pBLCFA and rabbit anti-CFA/I sera) or CFA/I subunits (MAb 84) as solid phase bound antigens. MAb 84 was previously diluted (1:25) with PBS before testing. Denatured CFA/I subunits were uses as solid-phase bound antigen for reactions carried out with MAb 84.

Table 1
Slide agglutination assays and IHA of anti-CFA/I antibodies tested against whole bacterial cells expressing CFA/I fimbriae

| Sera | Agglutination[a] | IHA[a] |
|---|---|---|
| CFA/I[b] | +++ | +++ |
| PBLCFA | + | ++ |
| PkCMVintBL | – | – |

[a] Degree of agglutination or inhibition of hemagglutination promoted by the CFA/I-positive strain: +++ (very strong), ++ (strong), + (weak), – (none).
[b] Positive control serum raised in rabbit immunized with intact CFA/I fimbriae.

Protective antibody responses against ETEC, as well as other enteric non-invasive pathogens, mainly require activation of local IgA production. In contrast to other vaccine approaches, as attenuated live vaccine strains, such responses are usually not induced by parentally administered DNA vaccines [10,39,40]. Attempts to elicit mucosal IgA responses with DNA vaccines have relied on shuttling by orally delivered attenuated *Shigella* or *Salmonella* strains [41,42], intranasal administration of DNA–lipid complexes or concomitant administration of plasmids encoding cytokines mediating local responses [43]. Recently, we have shown that mice i.m. inoculated with a DNA vaccine encoding a cell-associated CFA/I subunit can prime CFA/I-specific systemic and mucosal antibody responses following oral boosting with a CFA/I-expressing live attenuated *Salmonella* vaccine strain [44]. Thus, the possibility of inducing antibodies against epitopes involved with the adhesive properties of the CFA/I fimbriae with pBLFCA raises new opportunities for the development of combined vaccination protocols based on priming with DNA vaccine and boosting with CFA/I- expressing bacteria, as an approach to attain protective mucosal IgA responses.

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