

# New vaccine strategies against enterotoxigenic *Escherichia coli*. II: Enhanced systemic and secreted antibody responses against the CFA/I fimbriae by priming with DNA and boosting with a live recombinant *Salmonella* vaccine

M.O. Lásaro<sup>1</sup>,  
A.M.B. Alves<sup>1,2</sup>,  
H.C.R. Guillobel<sup>3</sup>,  
D.F. Almeida<sup>1</sup> and  
L.C.S. Ferreira<sup>1</sup>

<sup>1</sup>Laboratório de Fisiologia Celular, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil  
<sup>2</sup>Laboratório de Tecnologia Bacteriana, Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil  
<sup>3</sup>Departamento de Biofísica e Biometria, Instituto de Biologia, Universidade Estadual do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

## Abstract

The induction of systemic (IgG) and mucosal (IgA) antibody responses against the colonization factor I antigen (CFA/I) of enterotoxigenic *Escherichia coli* (ETEC) was evaluated in mice primed with an intramuscularly delivered CFA/I-encoding DNA vaccine followed by two oral immunizations with a live recombinant *Salmonella typhimurium* vaccine strain expressing the ETEC antigen. The booster effect induced by the oral immunization was detected two weeks and one year after the administration of the DNA vaccine. The DNA-primed/*Salmonella*-boosted vaccination regime showed a synergistic effect on the induced CFA/I-specific systemic and secreted antibody levels which could not be attained by either immunization strategy alone. These results suggest that the combined use of DNA vaccines and recombinant *Salmonella* vaccine strains can be a useful immunization strategy against enteric pathogens.

## Key words

- DNA vaccine
- ETEC
- CFA/I fimbriae
- *Salmonella* bivalent vaccine

## Correspondence

L.C.S. Ferreira  
Laboratório de Fisiologia Celular  
Instituto de Biofísica, UFRJ  
21941-590 Rio de Janeiro, RJ  
Brasil  
Fax: +55-21-280-8193  
E-mail: lcsf@ibccf.biof.ufrj.br

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## Introduction

Stimulation of the mammalian immune system by intramuscular (*im*) administration of plasmid DNA encoding heterologous antigens represents an exciting new immunization procedure which has been repeatedly shown to induce humoral and cellular im-

mune responses, some of them protective upon challenge, against several pathogenic viruses, bacteria and parasites (1-3). Nonetheless, one of the main limitations of DNA vaccines has been the lack of an induced mucosal response, i.e., IgA isotype immunoglobulins produced on mucosal surfaces and in exocrine gland secretions. Secreted IgA

represents the most efficient and effective immune barrier against a number of pathogens, such as enteric bacterial pathogens which use the intestinal mucosal surface to gain access to the mammalian host (4). This has led to the search for alternative experimental approaches to target DNA vaccines to mucosal immune system inductive sites such as direct intravaginal delivery (5), oral delivery of encapsulated DNA in biodegradable microspheres (6), particle bombardment of mucosal surfaces with gene gun-delivered DNA-coated gold particles (7,8) or nasal administration of DNA-liposome complexes (9).

The combined use of parenteral (whole cell antigens) and oral (live bacterial cells) immunizations has been reported to enhance systemic and mucosal antibody responses in mice vaccinated against *Shigella* (10). Using a similar immunization strategy we evaluated the priming of the mucosal immune system by a parenteral DNA vaccine encoding the colonization factor I antigen (CFA/I) fimbrial adhesin subunit of enterotoxigenic *Escherichia coli* (11,12) followed by oral immunization with a live recombinant *Salmonella* vaccine strain expressing the same enterotoxigenic *Escherichia coli* (ETEC) antigen (13). We show that the combined immunization regime results in enhanced production of the CFA/I-specific systemic and secreted antibody response.

## Material and Methods

### DNA vaccine

pRECFA, which encodes the ETEC CFA/I subunit fused with herpes simplex virus glycoprotein D1 (HSV gD1) under the control of Rous sarcoma virus (RSV) promoter, was used for the DNA immunizations. The construction of pRECFA and its induced immune response following administration in mice have been reported (11,12).

### Attenuated *Salmonella* vaccine

The *S. typhimurium* HG3 strain is a non-reverting aromatic-dependent (*aroA*) histidine-requiring mutant transformed with pCFA-1, which encodes the ETEC *cfab* gene, as previously described (13). Before administration to mice, the *Salmonella* vaccine strain was cultivated at 37°C in Luria broth in the presence of 0.5 mM IPTG for 4 h to induce expression of CFA/I. Cells were centrifuged, washed once and resuspended in phosphate buffered saline (PBS) to a final concentration of approximately  $10^{10}$  cfu/ml and used the same day. Expression of CFA/I by HG3 was confirmed by SDS-PAGE and Western blot.

### Immunization protocol and sample collection

Four- to six-week-old male BALB/c mice purchased from Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro) were used in all experiments. For DNA vaccination mice were inoculated *im* with two 100- $\mu$ g doses of pRECFA given two weeks apart as described (11). For immunization with *Salmonella*, mice were intragastrically inoculated using a blunt-tipped feeding needle with two doses of  $10^{10}$  cfu in 0.5 ml of PBS given one week apart. Two groups of five mice were submitted to the combined immunization regime: the first (denoted young mice) received the first *Salmonella* vaccine dose two weeks following the last DNA immunization, while the second group (denoted old mice) was vaccinated with the recombinant *Salmonella* strain one year following the second DNA dose. Control groups were immunized only with two doses of HG3 or pRECFA or with two doses of pRE4, which did not contain the *cfab* gene insert, followed by two HG3 doses. Mice were bled by retro-orbital puncture before inoculation (preimmune sera) and two weeks after the last DNA immunization. One week after the last administration

of HG3, animals were sacrificed and bled by cardiac puncture. Initially, serum samples were individually tested for reactivity against CFA/I subunits in an enzyme-linked immunosorbent assay (ELISA), then pooled and stored at  $-20^{\circ}\text{C}$  for subsequent analysis. For analysis of the mucosal immune response, feces were collected overnight and freeze dried. Fifteen pellets were homogenized in PBS (0.5 ml) and centrifuged at 10,000 rpm for 10 min at  $4^{\circ}\text{C}$ , and the supernatant was collected for CFA/I-specific IgG IgA ELISA.

#### Detection of antibodies by ELISA

ELISA was carried out with purified heat-denatured CFA/I subunits as a solid-phase bound antigen prepared as described (14). CFA/I-specific IgG and IgA ELISA were carried out by standard procedures using sera and fecal extracts from immunized mice, respectively (11,13). CFA/I-specific IgG titers were determined by end-point dilution in ELISA.

#### Results

Two groups of mice immunized *im* with two 100- $\mu\text{g}$  doses of pRECFA given two weeks apart presented systemic CFA/I-specific IgG titers which reached maximum levels two weeks after the last dose (average of  $10^4$ ), followed by a declining phase of 20 weeks and later by the stabilization of anti-

body levels with titers ranging from 1 to  $2.5 \times 10^3$ . In pRECFA-inoculated mice orally boosted with two doses of CFA/I-expressing live recombinant *Salmonella* HG3 cells 2 or 52 weeks after the last DNA dose, the anti-CFA/I serum IgG levels rose to  $2 \times 10^5$  and  $6 \times 10^4$ , respectively (Figure 1). Animals immunized with only two doses of HG3 or with pRECFA alone presented reduced CFA/I-specific systemic IgG levels in relation to those immunized with the combined DNA-primed/*Salmonella*-boosted protocol (Figure 1).

Mucosal anti-CFA/I IgA responses were monitored in both groups of vaccinated mice (young and old mice). As was the case for the systemic antibody responses, the combined use of DNA and recombinant *Salmonella* vaccines resulted in an enhanced secreted IgA response in fecal extracts (Figure 2). However, the booster effect of orally delivered CFA/I-expressing *Salmonella* was demonstrable only in young mice, i.e., those which were immunized two weeks after the last DNA dose. CFA/I-specific IgA levels in mice orally boosted with live *Salmonella* cells two weeks after the last DNA dose were at least five times higher than those observed in mice immunized with two doses of HG3 or pRECFA only. Mice immunized with two doses of HG3 had significantly higher CFA/I-specific IgA levels which did not differ from the values found in those immunized with the combined DNA-primed/

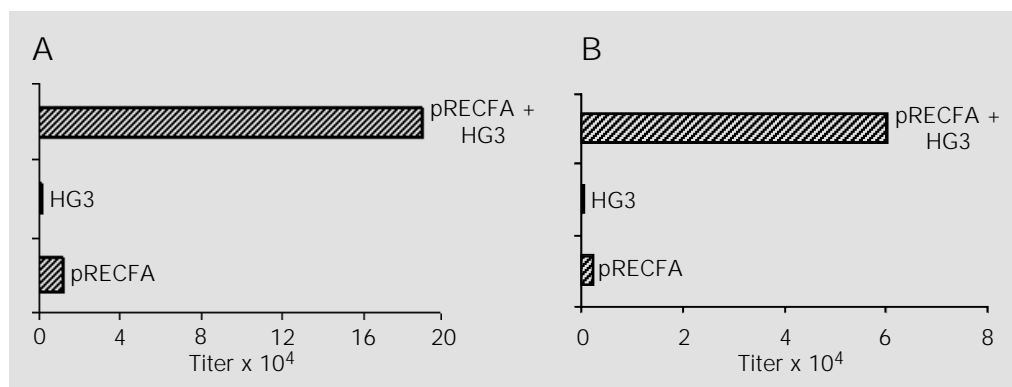
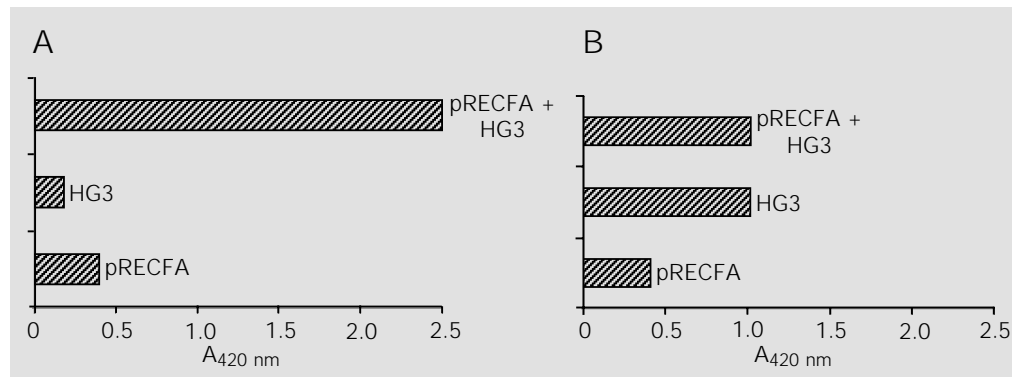


Figure 1 - Systemic IgG CFA/I-specific antibody response in mice immunized *im* with pRECFA as primer and orally boosted with two doses of *Salmonella* vaccine strain (HG3). The doses of *Salmonella* vaccine were given two weeks (A) or 54 weeks (B) after the last DNA dose. Mice were immunized with the complete protocol (pRECFA + HG3), two pRECFA doses (pRECFA) or two HG3 doses. Column length indicates the mean titers of pooled serum samples (N = 5). Standard deviations were less than 5% of the calculated mean values.

Figure 2 - CFA/I-specific secreted IgA (sIgA) antibody response in fecal extracts from mice immunized im with pRECFA as primer and orally boosted with two doses of *Salmonella* vaccine strain (HG3) detected by IgA-ELISA. The doses of *Salmonella* vaccine were given two weeks (A) or 54 weeks (B) after the last DNA dose. Mice were immunized with the complete protocol (pRECFA + HG3), two pRECFA doses (pRECFA) or two HG3 doses. Column length indicates the mean of duplicate absorbance values of pooled fecal extract samples (N = 5) at 1:4 dilution. Standard deviations were less than 5% of the calculated mean values.



*Salmonella*-boosted protocol in fecal extracts one year after the last dose (Figure 2).

In contrast to serum raised against CFA/I encoded by pRECFA (11), serum antibodies generated by the combined DNA-primed/*Salmonella*-boosted regime inhibited the adhesive properties of CFA/I fimbriae expressed by ETEC cells, as evaluated in a hemagglutination inhibition assay (data not shown), in contrast to serum CFA/I-specific antibodies induced by pRECFA which were not inhibitory under similar conditions (11).

## Discussion

In this study we analyzed the systemic and mucosal immune responses induced in mice immunized with a combined parenteral DNA-primed/oral *Salmonella*-boosted vaccination strategy. We observed, for the first time, that association of an *im* delivered DNA vaccine (primer) with a live recombinant attenuated *Salmonella* strain (booster) carrying a heterologous antigen (CFA/I) resulted in enhanced antigen-specific antibody production in both systemic (IgG) and mucosal (IgA) compartments. Moreover, our results indicated that, at least for systemic immune responses, the priming of the immune system by the DNA vaccine is a long-lasting event since inoculated animals were able to respond to the oral administration of *Salmonella* cells up to 52 weeks after DNA immunization.

It has been shown that parenteral immunization with heat-killed *Shigella* cells results in enhanced systemic and mucosal immune responses following oral boosting with live cells (10). In the present study we showed that the same rationale could be applied to DNA vaccines. Using a DNA vaccine encoding the CFA/I ETEC fimbrial adhesin to prime the animals and a CFA/I expressing *Salmonella* vaccine strain as the oral boosting formulation we detected antibody levels in both serum and fecal extracts, which could not be attained by only one immunization strategy. Thus, such approach overcomes in part the deficient activation of mucosal immune responses by *im* delivered DNA vaccines since activation of IgA production could be efficiently triggered in DNA vaccinated mice following local stimulation with recombinant live *Salmonella* cells. Although several recent reports have indicated that direct stimulation of the mucosal immune system is feasible (5-9), the combined vaccination strategy described here does not require any specialized delivery method and takes advantage of both immunization protocols, i.e., the strong and long-term stimulation of antibody responses induced by DNA vaccines and the efficient targeting and stimulation of the mucosal immune system by oral immunization with live *Salmonella* cells.

Expression of secreted bacterial proteins such as toxins and adhesins by host cells

transfected with DNA vaccines may lead to the production of altered proteins with regard to their native conformations. Such *in vivo* formed antigens may lead to the production of reduced antibody levels or inappropriate epitope specificity (11,15). In both cases the protective efficacy of the vaccine would be seriously affected. Mice immunized with pRECFA have been shown to induce anti-CFA/I serum antibodies unable to inhibit the adhesive properties of intact CFA/I fimbriae due to altered epitope specificity (11). On the other hand, mice immunized with the combined DNA-primed/*Salmonella*-boosted protocol developed a systemic antibody response able to block the adhesive properties of CFA/I<sup>+</sup> ETEC cells. These results suggest that the combination of DNA priming with oral *Salmonella* boosting could not only activate a quantitatively higher antibody response but also a qualitatively different response based on the epitope specificity of the induced antibodies.

Recently, ETEC was included by the

WHO among the top five enteric pathogens to be controlled by vaccination (16). At present only one ETEC vaccine, based on the oral delivery of an inactivated pool of ETEC strains expressing the most prevalent CFAs and purified heat-labile toxin  $\beta$ -subunit, is under testing for human use (17). The proposed combination of two different immunization strategies which could enhance both systemic and mucosal antibody responses against an important ETEC adhesin may represent a significant step towards the development of a vaccine against enteric bacterial pathogens.

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