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Immunization against the colonization factor antigen I of enterotoxigenic *Escherichia coli* by administration of a bivalent *Salmonella typhimurium aroA* strain

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Abstract

An expression plasmid (pCFA-1) carrying the *cfaB* gene that codes for the enterotoxigenic *Escherichia coli* (ETEC) fimbrial adhesin colonization factor antigen I (CFA/I) subunit was constructed and used to transform a derivative of the attenuated *Salmonella typhimurium aroA* vaccine strain SL3261 carrying an F'*lacI*^{q}. Treatment of the transformed strain with isopropyl-ß-D-thiogalactopyranoside (IPTG) resulted in elevated *in vitro* expression of the CFA/I subunit. Although flagellar function and lipopolysaccharide (LPS) synthesis were similar in both the parental and the recombinant strains, spleen colonization was reduced in the recombinant strain. All BALB/c mice parenterally inoculated with the recombinant strain developed significant anti-CFA/I and anti-LPS serum antibody titers ($P<0.05$). Moreover, 2 of 5 mice orally inoculated with the engineered *Salmonella* strain developed anti-CFA/I intestinal IgA ($P>0.05$) while 4/5 of the same mice developed anti-LPS IgA ($P<0.05$). The results indicate that the vaccine strain elicited an antibody response against the bacterial host both after oral and intravenous immunization while the response against the CFA/I antigen was significant only after inoculation by the intravenous route.

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- Colonization factor antigen I

Introduction

Enterotoxigenic*Escherichia coli* (ETEC) causes diarrheal disease with high morbidity and mortality rates among children in developing countries (1). It is also the infectious agent associated with traveller's diarrhea, which afflicts people travelling to places where the pathogen is endemic (1,2). ETEC relies on its ability to colonize the small intestine to cause disease. Once attached, ETEC produces enterotoxins, leading to massive losses of electrolytes and water by the enterocytes (3). ETEC adhesion is usually mediated by fimbriae, also known as colonization factor antigens (CFAs), which bind to

glycolipid receptors on the enterocyte surface. The CFA/I is one of the best characterized ETEC fimbriae and has a widespread occurrence in endemic areas, including South America (4-6). The CFA/I fimbriae contain a single protein subunit encoded by the plasmidial *cfaB* gene endowed with structural and functional (adhesion) roles (7).

Effective immunization against ETEC-associated diarrhea requires an anti-colonization mucosal IgA response (3). Studies with volunteers have shown that oral administration of large amounts of purified CFA/I fimbriae can induce protection against colonization by strains expressing CFA/I (8). However, protection afforded by purified CFA/I fimbriae is inefficient due to their degradation in the gastric and intestinal tracts (9). Alternatives to orally administered purified fimbrial preparations include direct delivery by orogastric instillation or the use of antigen-loaded biodegradable microspheres (10). However, difficult administration and/or purification costs turned out to be serious obstacles for the use of anti-ETEC vaccinal strategies based on isolated CFA/I fimbriae. A distinct approach to achieve an immune response against ETEC fimbriae relies on the oral administration of inactivated ETEC strains expressing epidemiologically relevant fimbriae, i.e., CFA/I, CFA/II and CFA/IV (11). Although anti-CFA-specific protective immunity could be evoked with the inactivated cellular ETEC vaccine, quality control, safety and low immunogenicity represent relevant and unsolved issues which preclude the large scale use of such vaccine formulation. Therefore, new antigen delivery systems and/or alternative antigen administration routes for fimbrial adhesins are mandatory issues for the development of efficient ETEC vaccines.

Attenuated *Salmonella* strains can invade the surface of the intestinal mucosa and induce a strong immune response without causing any damage to the host (12,13). Moreover, attenuated *Salmonella* strains can be used as vectors to deliver heterologous antigens of vaccine significance, thereby inducing cell-mediated immunity as well as mucosal and serum antibody responses against the *Salmonella* vector and the passenger antigen. A large number of bivalent *Salmonella*-based vaccines against a variety of pathogens have been developed, expressing antigens of viruses, bacteria and parasites (14-16). Previous reports have also shown that attenuated *Salmonella* strains can express surface-exposed ETEC fimbriae, such as K88, K99 and CFA/I, from cloned sequences encompassing the complete encoding operons (17-19). However, expression of fimbrial subunits as an intracellular antigen by a *Salmonella* vaccine strain and the subsequent activation of the immune system by different administration routes has not been attempted.

In the present study we report an alternative strategy of intracellular CFA/I subunit expression based on the use of an attenuated *S. typhimurium* strain carrying a double-plasmid regulatory system. Oral and intravenous administration of the recombinant *Salmonella* vaccine strain resulted in serum and mucosal immune responses against the *Salmonella* vector and the fimbrial subunit.

Material and Methods

Bacterial strains and plasmids

The non-reverting aromatic-dependent (*aroA*) histidine-requiring *Salmonella typhimurium* SL3261 vaccine strain has been described previously (12) and was kindly provided by Dr. B.A.D. Stocker, Stanford University School of Medicine. The *E. coli* K-12 strains D1210 and DH5α were employed in the selection of recombinant plasmid constructs. The ETEC strain TR 50/3 ($O63:H^-$, CFA/I^+, LT^+/ST^+) was isolated from a diarrheic child in São Paulo and was kindly supplied by Dr. B.E.C. Guth, Escola Paulista de Medicina (6). The pKK223-3 plasmid was purchased from Pharmacia. The DH5α

F'IQ strain (GibcoBRL, Gaithersburg, MD, USA) was used as donor of F', a plasmid that carries the *lacI*^{q} gene and codes for kanamycin resistance.

Media and growth conditions

Bacteria were grown at 37°C with shaking. Luria-Bertani broth (LB) containing 1% tryptone, 0.5% NaCl, and 1% yeast extract was supplemented with 10 µg/ml of 2,3-dihydroxybenzoic acid (DHB, Sigma Chemical Co., St. Louis, MO). When appropriate, growth media were complemented with ampicillin (100 µg/ml), streptomycin (70 µg/ml), kanamycin (25 µg/ml), or rifampicin (25 µg/ml). Spleen homogenates were plated onto brilliant green agar (BGA, Oxoid, Basingstoke, Hampshire, England) and xylose lysine deoxycholate agar (XLD, Oxoid) for detection of the *Salmonella aroA* strain, as previously described (20). Induction of *cfaB* expression was obtained with isopropyl-ß-D-thiogalactopyranoside (IPTG) (Sigma) at final concentrations ranging from 0.1 to 1 mM. For evaluation of cell motility, *Salmonella* strains were grown on 0.35% agar plates containing 0.65% tryptone (21).

Cloning of the *cfaB* gene and construction of the CFA/I overexpressing plasmid

Based on the available nucleotide sequence (22), forward (5'GGCCCGGGGGGAGGTCATTTCTAATGAAATTTAAAAAAACT3') and reverse (5'GGCCCGGGTCAGGATCCCAA3') PCR primers were used to amplify the structural *cfaB* gene, including a modified ribosome binding site, from purified total DNA extracted from the ETEC strain TR 50/3. A *Sma*I restriction site (underlined bases) was included in the 5' terminal of both primers. The PCR reactions were performed with *Taq* DNA polymerase using 30 temperature cycles of 1 min at 90°C, 1 min at 72°C and 1 min at 55°C. A 550-bp DNA band corresponding to the *cfaB* gene was purified from agarose gels, digested with *Sma*I, and ligated to the *Sma*I cleaved pKK223-3 plasmid. The ligation mix was used to transform *E. coli* D1210 and DH5α strains. Recombinant clones were selected by colony blotting using mouse anti-CFA/I subunit-specific serum and rabbit anti-mouse IgG-peroxidase conjugate antibody (Sigma) after growth on LB plates containing ampicillin and 0.5 mM IPTG. The recombinant plasmids were isolated and subjected to partial mapping with restriction enzymes. One plasmid, pCFA-1, with a correct insert size and proper orientation was chosen. The expression of the CFA/I subunit by the recombinant clones was determined by Western blot using CFA/I subunit-specific mouse antiserum and peroxidase conjugate antibody. Colony blots, plasmid DNA isolation, restriction enzyme digestion and ligation reactions were performed as described (23) or according to manufacturer instructions.

Construction of the *Salmonella* vaccine strain

F' *lacI*^{q} was conjugally transferred to a rifampicin-resistant mutant of *S. typhimurium* SL3261 by overnight mating with DH5α F'IQ on nitrocellulose filters (24). Transconjugants were selected on LB plates containing rifampicin and kanamycin at 37°C. Exponential growth phase *S. typhimurium* cells carrying F' *lacI*^{q} were transformed with pCFA-1 by electroporation using a gene pulser apparatus (Bio Rad, Hercules, CA, USA) and conditions recommended by the manufacturers. Clones harboring both plasmids were selected on ampicillin-kanamycin-containing plates and the plasmid contents were evaluated by the alkaline lysis procedure and by agarose gel electrophoresis (23). The clone selected for further use was denominated HG3.

Plasmid stability

In vitro plasmid stability was measured in LB broth in the absence of antibiotic selection.

IPTG (0.5 mM) was added to an aerated exponential growth phase HG3 culture (optical density (OD) of 30 Klett units) and incubated at 37°C. Samples were collected 4 and 24 h following IPTG addition, diluted in PBS and plated onto LB agar with or without ampicillin for viable cell counting.

Vaccine preparation

Exponential growth phase *S. typhimurium* HG3 and SL3261 strains were cultivated in LB containing ampicillin and streptomycin or streptomycin only, respectively. IPTG (0.5 mM) was added to HG3 cultures (OD of 30 Klett units) and incubated at 37°C for 4 h in a water bath shaker. Cells were centrifuged, washed once with PBS, suspended in PBS containing 10% glycerol to a final concentration of approximately 10^{10} colony-forming units (cfu)/ml and kept at -70°C until use.

Immunization protocol and sample collection

Five to 8 week-old male BALB/c mice were from the Instituto Nacional do Câncer (Rio de Janeiro). Bacterial cell suspensions were diluted in PBS and aliquots (100 µl) containing approximately 10^6 cfu were administered intravenously (*iv*) via the tail vein to groups of 5 mice. The groups were immunized with a single dose on day 0 and boosted with an identical dose 30 days later. The animals were sacrificed 20 days after the booster dose. On the day before immunization and on the day of sacrifice blood samples were collected by retroorbital or tail bleeding. Serum samples were harvested and kept frozen at -20°C until assayed. For oral immunization, groups of 5 mice were inoculated with 10^9 cfu at days 0 and 4 followed by an identical booster dose 26 days later. Twenty days following the booster dose, the small intestine (the segment between the pylorus and the ileocecal junction) was excised, sliced and homogenized in 50 mM EDTA and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF, Sigma). Samples were clarified by centrifugation, the supernatant was lyophilized, resuspended in 2 ml of TEAN buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 3 mM NaN_3, and 200 mM NaCl), and dialyzed overnight against TEAN buffer at 4°C. Samples were kept frozen at -20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Whole-cell extracts were analyzed by SDS-PAGE followed by Coomassie brilliant blue R-250 staining. Mass-normalized cell samples (about 10^7 exponentially growing cells) were suspended in electrophoresis sample buffer, boiled, and submitted to 15% PAGE (25). Proteins were transferred to nitrocellulose membranes and the CFA/I subunit was detected with mouse anti-CFA/I subunit serum and rabbit anti-mouse IgG-peroxidase conjugate antibody as previously described (21). Bound antibodies were developed with the ECL chemoluminescence system (Amersham International plc., Little Chalfont, Buckinghamshire, England). *Salmonella* LPS was detected in proteinase K-treated whole-cell extracts followed by silver staining of polyacrylamide gels (26).

ELISA

Serum and small intestine anti-CFA/I and anti-lipopolysaccharide (LPS) IgG responses were assayed on MaxiSorp microtiter plates (Nalge Nunc International, Naperville, IL, USA) previously coated with 0.1 µg/well of purified and heat-dissociated CFA/I fimbriae or 0.2 µg/well of *S. typhimurium* LPS (Sigma). After overnight blocking with 1% skim milk in PBS at 4°C, serum or gut homogenates, diluted in PBS-Tween 20 (0.05%), were added to the wells and incubated at room temperature for 90 min. Bound antibodies were detected with rabbit anti-mouse IgG-

(for sera) or rabbit anti-mouse IgA- (for gut homogenates) peroxidase conjugate antibodies (Sigma) and developed with orthophenylenediamine (Sigma) and H_2O_2 as enzyme substrate. Absorbance was measured at 490 nm in a Bio Rad model 450 microplate reader. The titers are indicated by the absorbance at 490 nm of 1:6,400 (LPS) and 1:400 (CFA/I) dilutions of sera or 1:20 dilution of the gut homogenates.

Spleen persistence of the *Salmonella* strains

Six BALB/c mice were injected *iv* with 10^6 cfu of HG3 or SL3261 strains. At days 1, 7 and 14 post-inoculation two animals of each group were sacrificed and the spleens removed. Spleens were homogenized separately in 1 ml of cold PBS in a mechanical Potter apparatus and aliquots (100 µl) plated onto BGA agar with and without ampicillin. When required spleen homogenates were diluted in PBS before plating. Plates were incubated overnight at 37°C for viable cell counts. Spleen samples were also spread on XLD agar plates to discriminate between the *Salmonella aroA* vaccine strain and wild type *S. typhimurium* contaminants. The *aroA* phenotype was detected by the translucent appearance of bacterial colonies on XLD agar plates while colonies of wild type strains were dark due to H_2S production.

Statistical analysis

The standard deviation of the mean was calculated for all ELISA absorbance data and colony forming units (spleen persistence and plasmid stability). The mean of variously immunized groups of mice were compared by the Student *t*-test.

Results

Construction of an *S. typhimurium aroA* vaccine strain expressing the CFA/I subunit

Attempts to introduce pCFA-1 by electroporation into *S. typhimurium* SL3261 or *E. coli* DH5α were unsuccessful, probably due to the toxic constitutive expression of the CFA/I subunit in the absence of the LacI repressor. Efficient repression of the *cfaB* gene and successful transformation of *Salmonella* cells with pCFA-1 were obtained after conjugal transfer of F' *lacI*^{q}. The HG3 clone harboring both plasmids and expressing high inducible levels of the CFA/I subunit (about 5% of total protein) was selected for further use (Figure 1A). Since there was

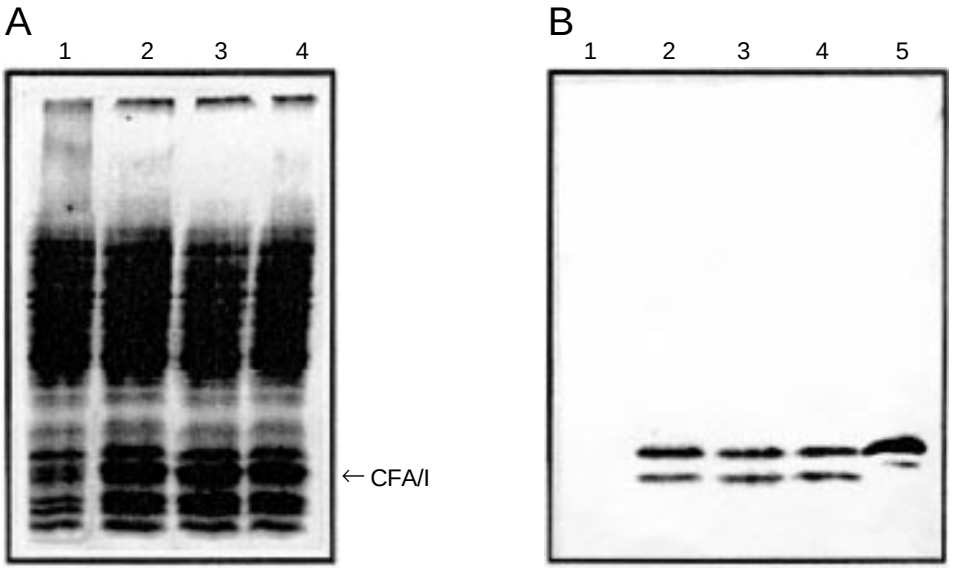

Figure 1 - Expression of the CFA/I subunit by the recombinant *S. typhimurium* HG3 strain. Whole-cell extracts (about 10^7 cells) of the HG3 strain were subjected to SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue (20 µg/well) (A), or submitted to Western blot analysis with anti-CFA/I subunit-specific serum diluted 1:2000 (B). *Lane 1*: non-induced HG3 cells; *lanes 2, 3* and *4*: HG3 cells induced with 0.1 mM, 0.5 mM and 1 mM IPTG, respectively; *lane 5:* 5 µg of purified CFA/I subunit.

no significant difference in the expression of the CFA/I subunit in cells induced by IPTG concentrations ranging from 0.1 mM to 1 mM, the concentration of 0.5 mM was chosen for further experiments (Figure 1A). The recombinant and native CFA/I subunits have similar electrophoretic mobilities with an apparent molecular weight of 15 kDa. One band of approximately 14 kDa, a probable degradation product, was observed in whole-cell extracts of IPTG-induced HG3 cultures and in purified CFA/I subunit preparations (Figure 1B). Immunoblots of subcellular fractions of IPTG-induced HG3 cells showed that the recombinant CFA/I subunit was found predominantly in the soluble fraction (cytoplasm and periplasm) of sonically disrupted cells (data not shown).

Phenotypic properties and plasmid stability of the *S. typhimurium* HG3 strain

No modification in LPS O antigen side chains of the HG3 strain could be detected in silver-stained polyacrylamide gels of proteinase K whole-cell digests (Figure 2). Similarly, no significant impairment of HG3 strain motility was detected in relation to the parental SL3261 strain, as evaluated by the size of swarming halos formed on soft agar plates after overnight growth (data not shown).

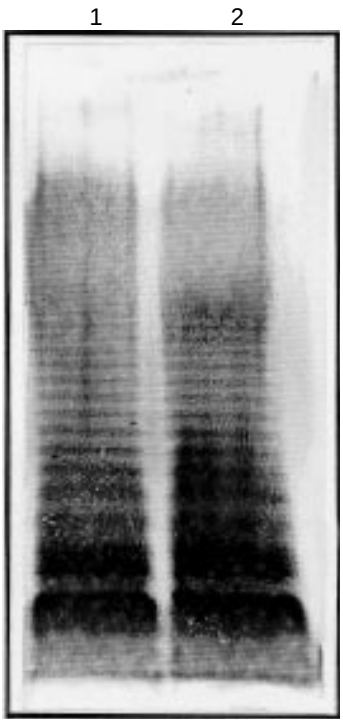

Figure 2 - Lipopolysaccharide composition of *S. typhimurium* SL3261 and HG3 strains. Bacteria grown in LB (about 10^7 cells) were treated with proteinase K and subjected to SDS-PAGE, followed by silver staining of LPS. *Lane 1 - S. typhimurium* SL3261; *lane 2 - S. typhimurium* HG3.

In the absence of ampicillin and IPTG there was no significant plasmid segregation during *in vitro* growth of the HG3 strain. All colonies tested after 24 h growth in LB medium were still resistant to ampicillin and were able to express the CFA/I protein when IPTG was added (data not shown). On the other hand, incubation of HG3 cells in the presence of 0.5 mM IPTG for 4 h resulted in a significant reduction (approximately 40%) of ampicillin-resistant cells which indicated segregation of the recombinant plasmid (Table 1). The number of ampicillin-sensitive colonies was increased to approximately 80% of the total counts when the incubation time was extended to 24 h (Table 1). As expected, only ampicillin-resistant colonies were able to express the CFA/I subunit when exposed to IPTG (data not shown).

Persistence and plasmid stability of the *S. typhimurium* HG3 strain during *in vivo* growth

The number of *Salmonella* organisms per spleen in animals which received a single *iv* dose of 10^6 cfu ranged from 1,000 cfu in those inoculated with HG3 to >10,000 cfu in those inoculated with SL3261 one week after injection (Figure 3). In both groups there was a slight increase in the number of *Salmonella* organisms per spleen two weeks after injection (Figure 3). Both strains were cleared from the spleens of inoculated mice one month after inoculation and all animals were apparently healthy at sacrifice (data not shown).

Immunization of mice with the *S. typhimurium* HG3 strain

All mice inoculated *iv* with either HG3 or

Table 1 - Evaluation of pCFA-1 stability in HG3 cells grown under inducing conditions.

[a]Exponential phase HG3 cells were incubated in the presence of 0.5 mM IPTG for 4 or 24 h. [b]Results are reported as the mean ± SD of quadruplicates from two identical experiments.

Inducing condition[a]	Number of viable cells (cfu/ml)[b]	
	LB	LB + ampicillin
-	$4.75 \pm 0.20 \times 10^9$	$4.52 \pm 0.27 \times 10^9$
4 h	$3.15 \pm 0.20 \times 10^8$	$1.91 \pm 0.20 \times 10^8$
24 h	$1.55 \pm 0.14 \times 10^9$	$3.70 \pm 0.80 \times 10^8$

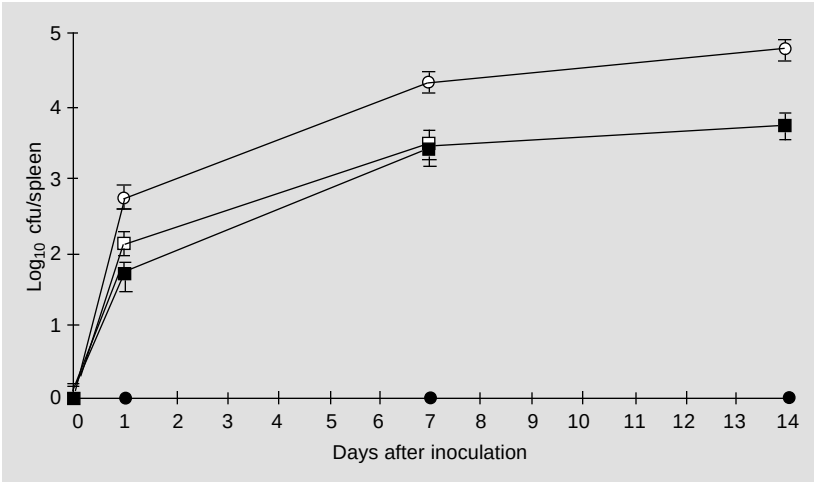

Figure 3 - Spleen persistence of *S. typhimurium* SL3261 and HG3 strains. BALB/c mice received a single *iv* dose of 10^6 cfu of SL3261 (circles) or 10^6 cfu of HG3 (squares). Mice were sacrificed and spleens removed at the times indicated. Spleen homogenates were plated onto BGA with (closed symbols) or without (open symbols) ampicillin at 100 µg/ml. Results are reported as the mean ± SD of quadruplicates from two identical experiments.

SL3261 had significant ($P<0.05$) serum anti-LPS IgG titers while non-immunized mice sera showed background ELISA values (Figure 4A). Similarly, mice immunized with HG3, but not those immunized with SL3261, had elevated ($P<0.05$) serum anti-CFA/I IgG levels while no significant reaction was found in the sera of non-immunized mice (Figure 4B).

Four of 5 mice orally immunized with HG3 had significant ($P<0.05$) anti-LPS IgA levels in gut homogenates whereas 5 of 5 mice immunized with SL3261 elicited an anti-LPS IgA response ($P<0.05$) (Figure 5A) and 2 of 5 mice immunized with HG3 showed an anti-CFA/I IgA response in gut homogenates ($P<0.10$) (Figure 5B). In contrast, orally administered SL3261 failed to elicit an anti-CFA/I IgA response (Figure 5B). Similarly, no anti-CFA/I IgA response was detected in a serum pool from non-immunized mice. These results indicate that the HG3 vaccine strain could elicit an antibody response against the host antigen after both oral and intravenous immunization while the response against CFA/I was significant only after intravenous inoculation.

Discussion

The use of attenuated *Salmonella* strains as vehicles to deliver heterologous antigens to the immune system represented an important step in the generation of orally administered bivalent vaccines (15). However, this approach faces two main difficulties. First, it is assumed that large initial amounts of the heterologous antigen should be accumulated by the vaccine strain in order to prime the host immune system, and second, sustained expression of the antigen seems to be required during the persistence time of the strain in host tissues (14,16,27). Plasmid expression is usually employed to attain high antigen levels but the metabolic overload or toxicity associated with excessive accumulation of the heterologous antigen may be deleterious to the vaccine strain. In the present study, for instance, the constitutive expression of the *cfaB* gene under the control of a strong promoter was lethal to the bacterial cell. The use of a double-plasmid regulatory system composed of a *tac* promotor-controlled *cfaB* gene-encoding plasmid and a second plasmid encoding the LacI repressor allowed the generation of a high antigen load without seriously affecting the viability of the strain or the expression of phenotypic properties relevant for the immunogenic potency. The presence of the recombinant plas-

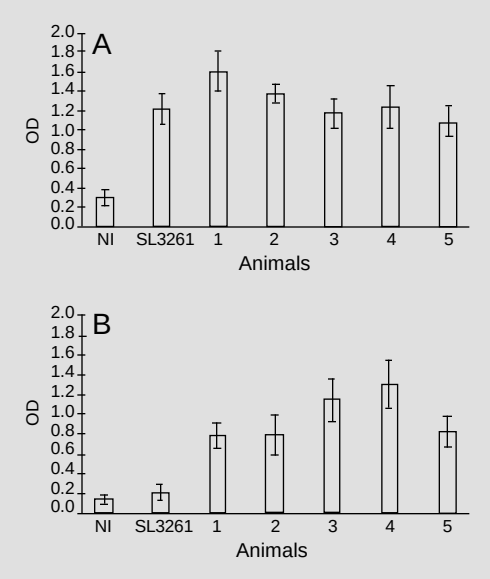

Figure 4 - Serum antibody response of mice immunized *iv* with *S. typhimurium* HG3 cells. The serum samples of 5 mice immunized with 10^9 cfu of HG3 were analyzed individually (numbers 1 to 5) by IgG-ELISA for *Salmonella* LPS at a final dilution of 1:6,400 (A) and the CFA/I subunit at a final dilution of 1:400 (B). Results obtained with pools of non-immunized (NI) and SL3261-inoculated (SL3261) mouse sera are also shown. Results are reported as the mean ± SD of quadruplicate wells of two identical experiments.

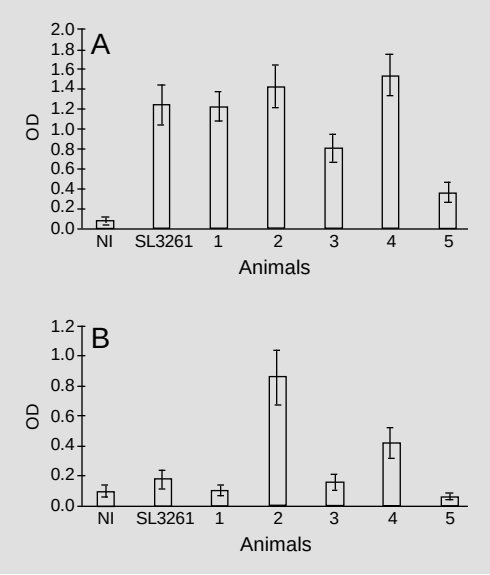

Figure 5 - Secreted antibody response of mice orally immunized with *S. typhimurium* HG3 cells. The gut homogenates of 5 mice immunized with 10^9 cfu of HG3 were analyzed individually (numbers 1 to 5) by IgA-ELISA for *Salmonella* LPS (A) and the CFA/I subunit (B). Results obtained with pools of non-immunized (NI) and SL3261-inoculated (SL3261) mouse gut homogenates are also shown. All samples were diluted 1:20 in PBS before testing. Results are reported as the mean ± SD of quadruplicate wells for two identical experiments.

mid did not inhibit the ability of the *Salmonella* strain to persist in spleens of inoculated mice and, more relevant, the present data show that the activation of systemic and secretory immune responses could be triggered in *iv* and orally inoculated mice, respectively.

CFA/I fimbria expression in ETEC cells is a rather complex process and depends on environmental factors such as composition of the growth medium, temperature and growth stage of the cells. This fact is reflected by the requirement of at least 5 genes acting at the transcriptional or post-transcriptional level for expression of the *cfaB* gene and assemblage of CFA/I fimbriae (28). Therefore, instead of using fimbriated cells generated by the introduction of the whole CFA/I operon in the *Salmonella* strain, we cloned and expressed the CFA/I subunit structural gene under control of a strong inducible promoter leading to high intracellular levels of the antigen. The expression of cell-associated unpolymerized CFA/I subunits by the *Salmonella* vaccine strain may be advantageous for the induction of systemic and secreted antibodies since the antigen is protected from the harsh gastric and enteric environments which can degrade surface-exposed fimbriae. Moreover, elevated levels of CFA/I subunit could be easily obtained and controlled during *in vitro* growth of the vaccine strain, permitting a better standardization of the antigen load. Finally, dissociated CFA/I subunits, but not intact fimbriae, expose immunorecessive epitopes, which confer cross-reaction with other ETEC fimbriae such as CS1, CS2, CS4, CS17 (29-31). Taking into account all of these facts, the ability of the present HG3 vaccine strain to induce anti-CFA/I subunit-specific antibodies may represent an important step toward the generation of a broad-range anti-colonization ETEC vaccine.

It has been suggested that the initial load rather than the antigen synthesized during persistence of the bacterial cells in the host tissues is the most critical event for priming the gut-associated lymphoreticular tissue (GALT) and activation of the host immune response against the heterologous antigen delivered by orally administered recombinant *Salmonella* vaccine strains (27). The development of the *S. typhimurium* HG3 strain allowed us to obtain high initial CFA/I subunit levels but presumably the recombinant bacteria could not express additional amounts of the heterologous antigen after

administration to the host. Therefore, expression of the heterologous antigen in host tissues was apparently not essential for the activation of the systemic response since all mice immunized *iv* with the HG3 strain showed elevated anti-CFA/I subunit-specific IgG titers. In contrast, only 2 of 5 mice orally immunized with the HG3 strain developed detectable anti-CFA/I subunit-specific secreted IgA levels in their guts. The lack of a more efficient mucosal immune response may reflect the lack of *in vivo* CFA/I subunit expression by the *Salmonella* cells colonizing the Peyer's patches of vaccinated mice. Future experiments should address the question of how the priming and subsequent colonization of the mucosal immune system by the recombinant HG3 strain could contribute to the activation of a secretory immune response. Further improvements of the activation of the mucosal immune response may require continuous low level expression of the heterologous antigen or co-administration of a mucosal adjuvant.

Since the initial observations of Yamamoto et al. (32), surface expression of intact CFA/I fimbriae by recombinant *Salmonella* vaccine strains has represented one possible way to raise a specific immune response against the ETEC adhesin (17,19). The present results show that intracellular expression of CFA/I subunits by an attenuated *Salmonella* strain is an alternative approach to the problem of antigen presentation which may become a new strategy for the development of anti-*Salmonella*/ETEC bivalent vaccines.

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