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Immune Responses to Novel *Escherichia coli* and *Salmonella typhimurium* Vectors That Express Colonization Factor Antigen I (CFA/I) of Enterotoxigenic *E. coli* in the Absence of the CFA/I Positive Regulator *cfaR*

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An *asd*-stabilized plasmid carrying enterotoxigenic *Escherichia coli cfaABCE* genes was constructed and called pJGX15C-*asd*$^+$. Expression of colonization factor antigen I (CFA/I) by this plasmid occurs independently of the *cfaABCE* positive regulator *cfaR* in attenuated *Salmonella* Δ*aro* Δ*asd* strain H683 and nonpathogenic laboratory *E. coli asd* strain χ6212. Oral immunization of mice with nonpathogenic *E. coli* χ6212 (pJGX15C-*asd*$^+$) does not elicit significant serum or mucosal responses against CFA/I. In contrast, oral immunization with a single dose of attenuated *S. typhimurium* H683(pJGX15C-*asd*$^+$) elicits a 10^5-fold increase in CFA/I-specific serum immunoglobulin G and significant elevation of CFA/I-specific immunoglobulin A-secreting B cells in the lamina propria, mesenteric lymph nodes, and spleen. Thus, only the *Salmonella*-CFA/I construct effectively delivered CFA/I to the inductive sites of the gut-associated and systemic lymphoid tissues.

Infection with enterotoxigenic *Escherichia coli* (ETEC) ranks high as a public health problem in developing countries and to travelers from developed countries who visit regions where ETEC is endemic (22, 23). Currently, there is a need to develop a safe and effective ETEC vaccine to serve as a public health tool for the prevention of ETEC infection (20, 22, 33, 37). Purified ETEC colonization factor antigens (CFAs), which mediate attachment to enterocytes in the small intestine (7, 8), stimulated protective antibodies in animal models (6). Volunteers who ingested a purified preparation of ETEC fimbriae, however, did not develop an effective mucosal immune response (24), probably because of denaturation and degradation of the antigen by gastric acidity and proteases (29). Effective immunization of volunteers with purified ETEC fimbriae was achieved, though, when this antigen was delivered directly to the intestine by orogastric intubation (24). This clinical study demonstrated the need for a means to effectively deliver ETEC CFAs to the mucosal immune system of the intestine.

One solution is to express CFA in a live oral *Salmonella* vaccine vector (12, 37). Studies with *Salmonella* organisms expressing porcine ETEC K88 fimbriae demonstrated that such vaccine vectors elicit mucosal and serum antibodies against the fimbrial antigen (16, 30). However, *Salmonella* organisms, which are thought to reside predominantly in macrophages (1, 2, 25, 26), may be exposed to environmental signals in the host different from those to which ETEC, which colonizes the brush border of enterocytes and remains on the lumenal side of the small intestine, is exposed (7). Thus, the *cfa* genes may not be expressed by *Salmonella* vectors in the host if they are placed under the control of the native ETEC CFA/I positive regulator, called *cfaR* (28). Since in vivo expression of foreign antigens by bacterial vaccine vectors is considered important (4), we constructed plasmid pJGX15C, which expresses CFA/I in the absence of *cfaR* (36).

Antigen stability is another important consideration for the construction of *Salmonella* vaccine vectors (11, 16). To stabilize plasmids in *Salmonella* strains in the absence of antibiotic selection, Galan et al. (11) constructed a balanced lethal plasmid stabilization system in which a plasmid-based *asd*$^+$ allele complements a lethal chromosomal *asd* deletion in the recipient *Salmonella* strain. In this report we describe the construction of an *asd*-stabilized plasmid capable of *cfaR*-independent CFA/I expression in *Salmonella typhimurium* and *E. coli*. We then compared the immunogenicities of these CFA/I-expressing constructs in orally immunized mice.

Others have shown that five genes in two regions are necessary for high-level expression of CFA/I (35). The structural genes for CFA/I fimbria biogenesis, *cfaABCE*, are located in region 1, whereas region 2 encodes an Rns-like DNA-binding protein called CfaR (28), which positively regulates the expression of region 1.

On the basis of the published nucleotide sequence of *cfaABCE* in strain H10407 (15, 19), we designed forward (H60; 5′-TGGGAGCTCAGGAGGAAATATGCA-3′ [100 bp of upstream sequence, *cfaA*]) and reverse (H63; 5′-TGGCTC GAGCTAGAGTGTTTGACT-3′ [*cfaE*, stop codon, and *Xho*I site]) PCR primers for the amplification of these genes from purified wild-type ETEC strain H10407 (CFA/I$^+$ and positive for heat-labile enterotoxin; O78:H11) plasmid DNA (7). Using PCR primers H60 and H63 and H10407 plasmid DNA as a template, a PCR-generated fragment encoding *cfaABCE* was amplified after 30 PCR cycles (2 min at 45°C to anneal the primers and 10 min at 72°C for elongation) in standard PCR buffers with *Taq* polymerase (18). This fragment was digested with *Sac*I and *Xho*I, which produced a 5.2-kb fragment encoding from 10 bp upstream of *cfaA* to the end of *cfaE* and flanked by *Sac*I- and *Xho*I-compatible ends. We inserted this fragment

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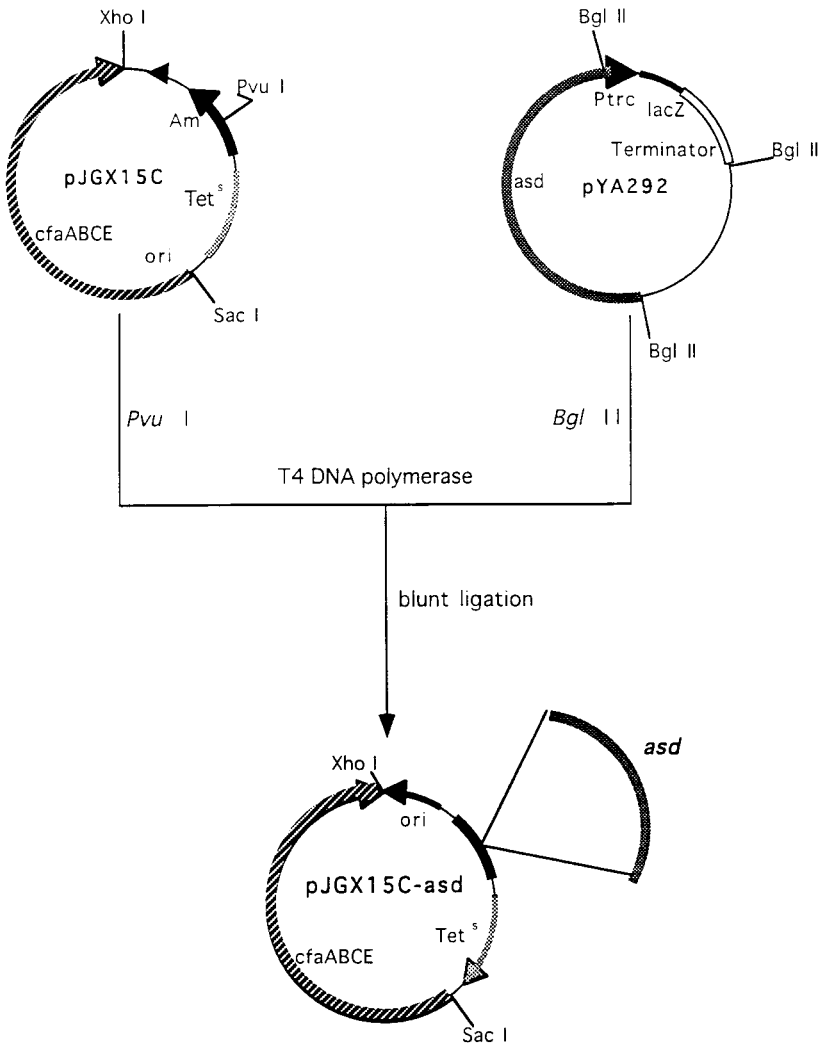

FIG. 1. Construction of pJGX15C-*asd*$^+$. To construct an *asd*$^+$-stabilized derivative of pJGX15C, a *Bgl*II fragment containing the *asd*$^+$ allele from pYA292 was inserted into the *Pvu*I site of pJGX15C. The derivative, pJGX15C-*asd*$^+$, is tetracycline and ampicillin sensitive and stably expresses CFA/I in *asd* strains *S. typhimurium* H683 and *E. coli* χ6212. ori, origin of replication.

into *Sac*I-*Xho*I-digested pJRD184, resulting in plasmid pJGX15A, which expressed CFA/I in the absence of *cfaR* in *E. coli* HB101 (12).

Since this plasmid does not encode the *cfa* promoter region, it seemed likely that the expression of CFA/I by strains bearing pJGX15A was probably under the control of the tetracycline resistance gene. To confirm this hypothesis we deleted the *Eco*RI-to-*Hin*dIII region in pJRD184 (using mung bean nuclease and blunt-end ligation) (27), which encodes most of the promoter region for the tetracycline resistance gene. The resultant plasmid, called pJRD184-Tc^s, is tetracycline sensitive. The 5.2-kb fragment encoding *cfaABCE* was subcloned from pJGX15A into pJRD184-Tc^s, resulting in plasmid pJGX15B. In line with our expectation, clone pJGX15B does not express significant levels of CFA/I fimbriae in HB101. To our surprise, however, colony immunoblots revealed that one clone (of 400) from the same experiment expressed extremely high levels of CFA/I even in the absence of *cfaR*. The plasmid in this isolate was called pJGX15C and is more extensively described elsewhere (36). Expression of CFA/I by pJGX15C was mapped to a region within the tetracycline resistance gene, about 1 kb upstream of *cfaA*, and was not due to low-level expression of the tetracycline resistance gene (36).

Because of environmental concerns, it is considered inappropriate to use antibiotic resistance markers in candidate human live-vaccine vector strains (11). To stabilize pJGX15C without antibiotic selection we inserted the *asd* gene from pYA292 (11) into pJGX15C, resulting in plasmid pJGX15C-*asd*$^+$ (Fig. 1). This was accomplished by inserting an end-filled *Bgl*II fragment from pYA292 into the *Pvu*I site in pJGX15C by blunt-end ligation. Restriction endonuclease digestion, ligation, and plasmid DNA preparations were performed by standard techniques (27). *E. coli* Δ*asd* mutant strain χ6212 (11) and *S. typhimurium* Δ*aroA* Δ*asd* mutant strain H683 (9) served as hosts of *asd*$^+$ plasmids. These strains were grown on nutrient agar (Difco, Detroit, Mich.) or nutrient broth (Difco) supplemented with diaminopimelic acid (50 μg/ml). The recombinant plasmids were introduced into χ6212 and H683 by electroporation by using a Gene Pulser set at 200 Ω, 25 μF, and 2.5 kV (Bio-Rad Laboratories, Hercules, Calif.) (17). Selection for transformants was achieved by growth on nutrient agar without diaminopimelic acid supplementation (neither χ6212 nor H683

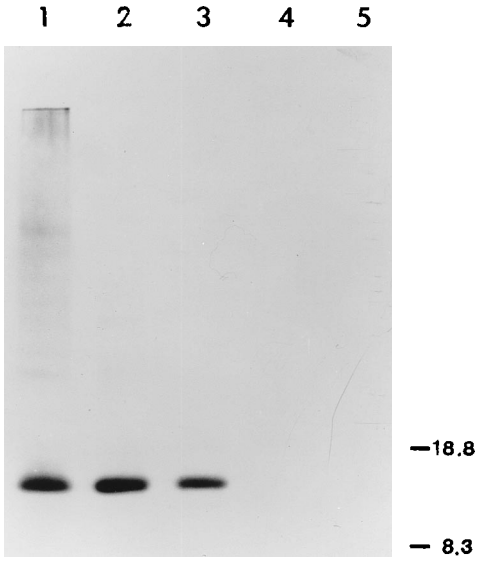

FIG. 2. Comparative immunoblot analysis of various CFA/I-expressing strains. CFA/I fimbriae were prepared from 10^8 CFU of wild-type ETEC H10407 (1), H683(pJGX15C-*asd*$^+$) (2), χ6212(pJGX15C-*asd*$^+$) (3), H683(pJRD184-*asd*$^+$) (4), and χ6212(pJRD184-*asd*$^+$) (5) as described in the text. These CFA/I samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with CFA/I-specific rabbit antiserum by a standard immunoblot technique (27). The results show that H683(pJGX15C-*asd*$^+$) and χ6212(pJGX15C-*asd*$^+$) produce similar levels of CFA/I. Numbers at the right are molecular masses in kilodaltons.

grows on this medium unless an *asd*$^+$ allele is supplied in *trans*) (11).

Using colony immunoblots (27), we found that CFA/I expression by pJGX15C-*asd*$^+$ in H683 and χ6212 was stable when these strains were grown on solid or in liquid media (data not shown). Comparative immunoblot analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated CFA/I preparations from H683(pJGX15C-*asd*$^+$) and χ6212(pJGX15C-*asd*$^+$) was performed essentially as described previously (14). In brief, the bacterial strains were incubated at 37°C for 16 h on colonization factor agar (18). Wild-type ETEC strain H10407 (7) was used as a CFA/I positive control. Bacteria were harvested by wiping the plates with a sterile cotton wool swab, and the bacteria were suspended in phosphate-buffered saline (PBS). The optical densities of these suspensions were adjusted so that there were 10^8 CFU/ml. Then, 1 ml of each adjusted suspension was placed into a microcentrifuge tube and centrifuged at 12,000 × *g* for 5 min. The bacterial pellets were resuspended in 40 μl of 1× SDS-PAGE loading buffer and boiled for 5 min. After boiling, bacterial cells were removed by centrifugation and 20-μl samples of the cell-free supernatants, containing solubilized CFA/I pilin subunits, were loaded onto an SDS–15% (wt/vol) polyacrylamide gel and electrophoresed at 80 mV for 2 h. After electrophoresis, the proteins were transferred (10 mV for 16 h) from the SDS-polyacrylamide gel to 0.2 μM nitrocellulose membranes (Bio-Rad).

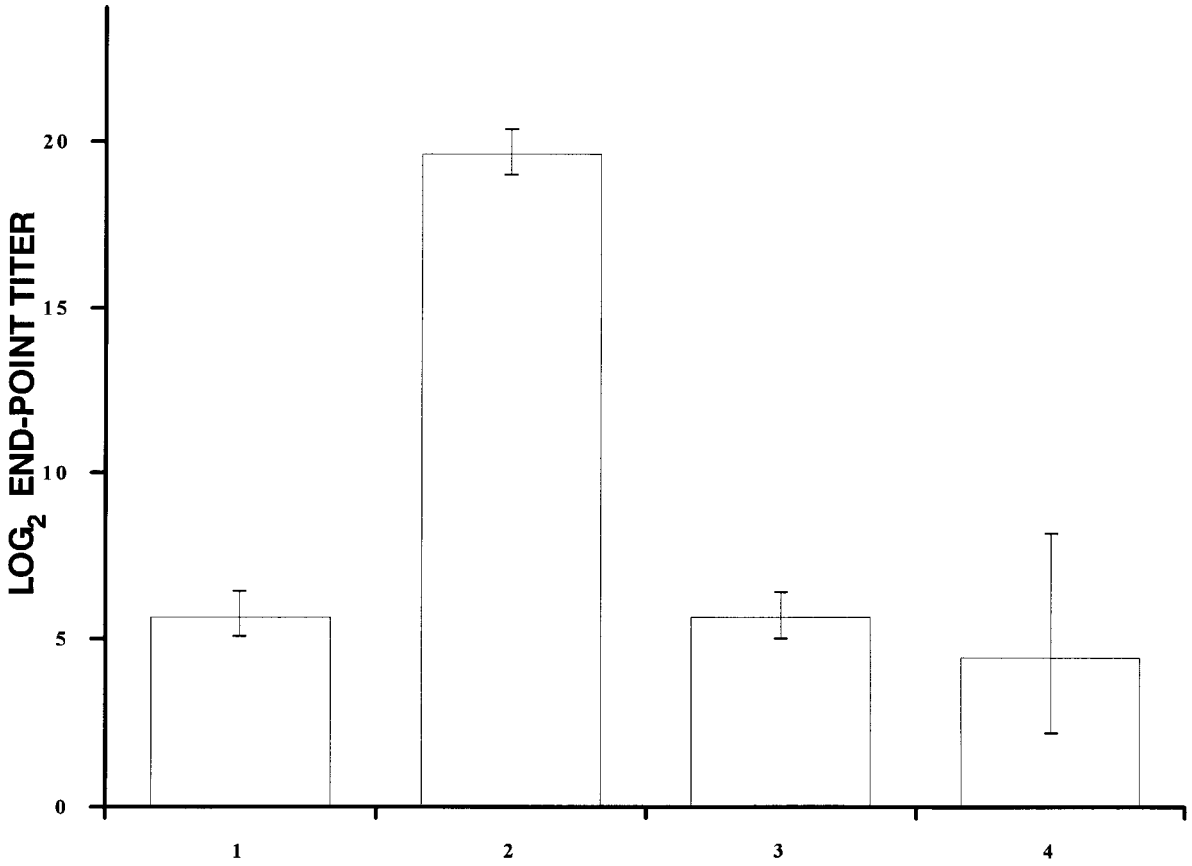

FIG. 3. CFA/I-specific serum IgG responses after oral immunization of mice. Shown are the geometric means ± the standard errors of the CFA/I-specific serum IgG $\log_2$ endpoint titers that developed after oral immunization of mice with a 10^9-CFU dose containing H683(pJRD184-*asd*$^+$) (1), H683(pJGX15C-*asd*$^+$) (2), χ6212(pJRD184-*asd*$^+$) (3), or χ6212(pJGX15C-*asd*$^+$) (4).

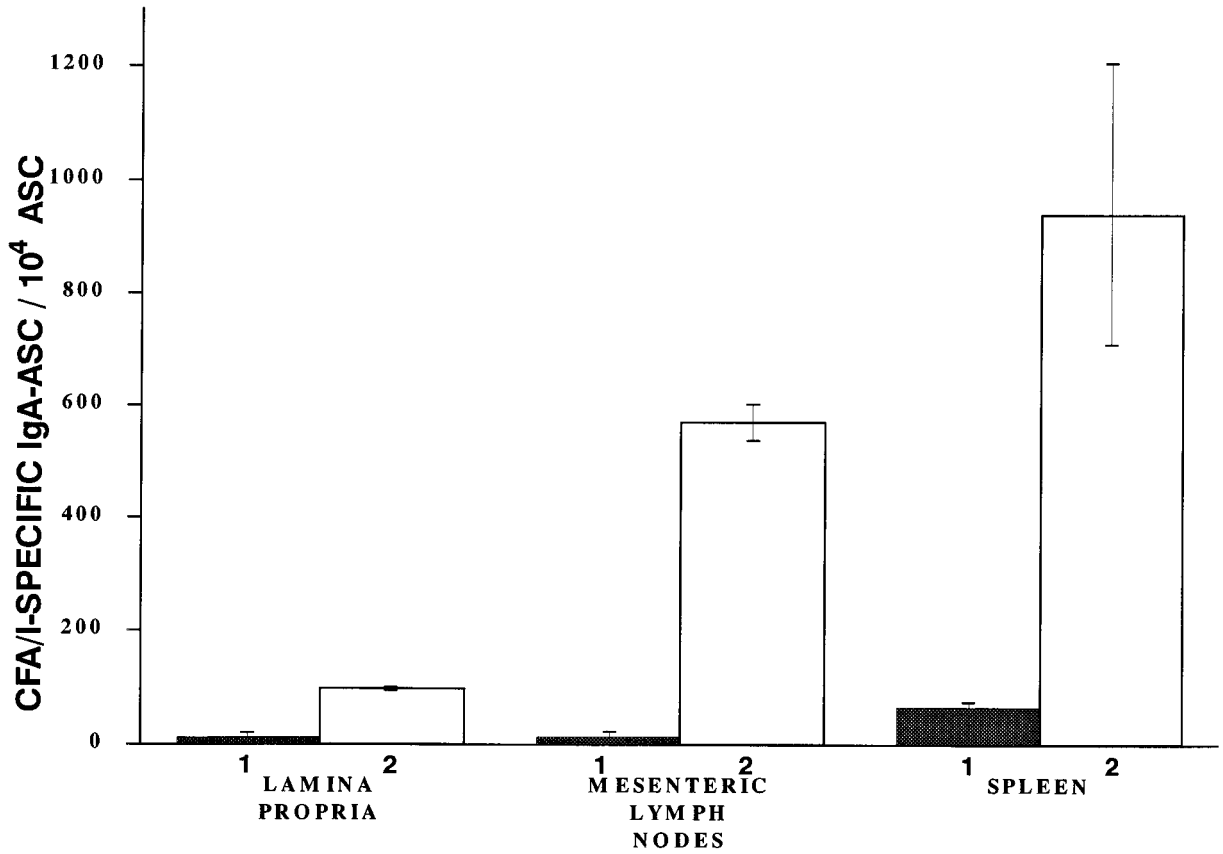

FIG. 4. CFA/I-specific serum IgA ASC responses after oral immunization of mice. CFA/I-specific IgA ASC per 10^4 IgA-ASC in LP, MLN, and spleen lymphocyte populations were measured by ELISPOT (3, 34) 28 days after oral immunization. Shown are the geometric means ± the standard errors of the CFA/I-specific IgA ASC per 10^4 IgA ASC that developed after oral immunization of mice with a 10^9-CFU dose containing H683(pJRD184-*asd*$^+$) (1) or H683(pJGX15C-*asd*$^+$) (2).

The membranes were probed first with primary antibody (HB101-absorbed rabbit polyclonal anti-CFA/I serum raised with purified CFA/I from H10407 [14]) and then with secondary antibody (goat anti-rabbit immunoglobulin G [IgG] conjugated to horseradish peroxidase [Amersham, Arlington Heights, Ill.]). Detection of primary-antibody-reactive protein bands was achieved by using a chemiluminescence detection system (Amersham). Analysis of H683(pJGX15C-*asd*$^+$) and χ6212(pJGX15C-*asd*$^+$) showed that these strains expressed similar levels of CFA/I (Fig. 2). Human erythrocyte hemagglutination (8) confirmed this observation (data not shown).

To assess the immunogenicity of the *Salmonella* and *Escherichia* recombinants, groups of five BALB/c mice each were immunized once orally with 5×10^9 CFU of H683(pJGX15C-*asd*$^+$) or χ6212(pJGX15C-*asd*$^+$) as described previously (16). Additional groups of five BALB/c control mice each were immunized orally with a single 5×10^9-CFU dose of either vector control H683(pJRD184-*asd*$^+$) or χ6212(pJRD 184-*asd*$^+$). Prior to each animal immunization experiment, the working seeds of these strains were grown at 37°C for 16 h on nutrient agar (Difco). Isolated colonies were aseptically transferred from the plates to 5 ml of normal saline (0.85% [wt/vol] NaCl). The saline suspensions were centrifuged at 8,000 × *g* for 8 min, and the supernatants were discarded. The bacterial pellets were resuspended in 1 ml of normal saline. The bacterial densities in the inocula were adjusted by using optical densities at 600 nm, and the viability of each inoculum for each experiment was determined in triplicate on solid medium (16). To immunize mice orally, the mice were given 0.2 ml of the appropriate bacterial suspension, containing about 10^9 CFU, by orogastric intubation (16).

To measure serum IgG responses to CFA/I, sera were collected before and 28 days after immunization. About 400 to 500 μl of blood was collected into individual tubes from the tail vein of each mouse and allowed to clot by incubation for 16 h at 4°C. After centrifugation in a microcentrifuge for 5 min, the serum samples were transferred to fresh tubes and stored at −20°C. Serum IgG responses to CFA/I in these serum samples were quantitated by enzyme-linked immunosorbent assay (ELISA) (33). Purified CFA/I fimbrial antigen was prepared as described previously (14), suspended in PBS (pH 7.2) at a concentration of 1 μg/ml, and used to coat 96-well ELISA plates (Maxisorp; Nunc). Goat anti-mouse IgG conjugated to horseradish peroxidase (1 μg/ml; Southern Biotechnology Associates, Birmingham, Ala.) was used to detect CFA/I-specific serum IgG. Endpoint titers were calculated by taking the inverse of the last serum dilution giving an absorbance of ≥0.1 optical density unit above the optical density at 405 nm of negative controls after 30 min of incubation (35).

The ELISA results indicated that H683(pJGX15C-*asd*$^+$) elicited a $>10^5$-fold increase in CFA/I-specific serum IgG response, with an endpoint titer of about 8.5×10^5 (Fig. 3). In contrast, no significant elevation in CFA/I-specific serum IgG was detected in mice immunized orally with noninvasive *E. coli*-CFA/I construct χ6212(pJGX15C-*asd*$^+$) or the vector control strains H683(pJRD184-*asd*$^+$) and χ6212(pJRD184-*asd*$^+$) (Fig. 3).

Lamina propria (LP) and mesenteric lymph node (MLN)

antigen-specific isotype-specific antibody-secreting cells (ASC) correlate with the development of mucosal humoral immunity (3, 34). Therefore, to obtain a measure of mucosal immunity to CFA/I, LPs and MLNs were harvested, by procedures that preserve lymphocyte function, 28 days after immunization (3, 34). Splenic lymphocytes were also harvested. By using standard IgA-specific ELISPOT assays the numbers of LP, MLN, and spleen CFA/I-specific IgA ASC were quantitated (3, 34) (Fig. 4). ELISA plates were coated with purified CFA/I (10 μg/ml) (14, 33) or tetanus toxoid (10 μg/ml) as a control antigen (3, 34). Results were expressed as the number of CFA/I-specific IgA ASCs per 10,000 IgA ASC (3, 34). In agreement with the serum IgG data, H683(pJGX15C-*asd*$^+$) induced a significant CFA/I-specific IgA ASC response in the LP, MLN, and spleen compared with the vector control H683(pJRD184-*asd*$^+$) (Fig. 4). *E. coli* χ6212(pJGX15C-*asd*$^+$), on the other hand, did not elicit measurable CFA/I-specific IgA ASC responses in these sites (data not shown).

Since the initial proposal to use *Salmonella* organisms to deliver CFA/I (37), construction of such recombinants that stably express high levels of CFA/I has been a significant obstacle. Strain H683(pJGX15C-*asd*$^+$) is a breakthrough in that this is the first *Salmonella*-CFA/I vaccine vector to stably express CFA/I in the absence of *cfaR*. Moreover, H683 (pJGX15C-*asd*$^+$) is the first *Salmonella*-CFA/I recombinant to elicit strong responses against CFA/I after immunization of mice with a single oral dose.

The data presented here show that oral immunization of mice with a single dose of *S. typhimurium* H683(pJGX15C-*asd*$^+$) elicited a 10^5-fold increase in CFA/I-specific serum IgG and IgA-secreting B cells in the LP and MLNs. In contrast, nonpathogenic *E. coli* χ6212(pJGX15C-*asd*$^+$) did not elicit significant serum or mucosal responses against CFA/I. Thus, the responses induced by the *Salmonella* construct probably cannot be explained in simple terms. Previously, de Aizpurua and Russell-Jones (5) showed that the immunogenicity of antigens given orally correlates with the intestine-binding properties of the antigen, where antigens that bind to the intestinal surface display the greatest immunogenicity. Furthermore, attachment to and invasion of the intestine by *Salmonella* organisms appear to preferentially occur at the Peyer's patches (10, 21). An explanation for our results, therefore, is that H683(pJGX15C-*asd*$^+$), which is capable of crossing the intestinal epithelial surface and persisting within the gut-associated lymphoid tissue and reticuloendothelial system for a limited period, delivers significantly more antigen to inductive sites of the gut-associated and systemic lymphoid tissues than *E. coli* χ6212(pJGX15C-*asd*$^+$). Alternatively, since χ6212(pJGX15C-*asd*$^+$) produces rough lipopolysaccharide, this strain may not persist in the host tissue, which would make it poorly immunogenic. Whatever the explanation, the data presented herein clearly demonstrate that live oral *Salmonella* organisms are a highly effective vector vehicle for delivery of CFA/I.

Recently, a double *aro* mutant of *Salmonella typhi* Ty2, called CVD 908 (17), was shown to be well tolerated in phase 1 volunteer studies (31, 32). Furthermore, oral immunization of volunteers with a single 5×10^7-CFU dose of CVD 908 induces *Salmonella*-specific intestinal IgA (measured by ELISPOT counts of antigen-specific ASC in peripheral blood mononuclear cells shortly after immunization) and *Salmonella*-specific serum IgG responses (13, 31, 32). The data presented here for the mouse typhoid model and an *S. typhimurium* Δ*aro* vaccine vector expressing CFA/I support the proposal to use Δ*aro S. typhi* CVD 908 as a vaccine vector for the delivery of CFA/I to the gut-associated lymphoid tissue in humans and to stimulate protective immunity against homologous CFA/I-expressing ETEC.

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