Antibody-Secreting Cells in Human Peripheral Blood after Oral Immunization with an Inactivated Enterotoxigenic Escherichia coli Vaccine

CHRISTINE WENNERÅS, ANN-MARI SVENNERHOLM, CHRISTINA ÅHRÉN, AND CECIL CZERKINSKY*

Department of Medical Microbiology and Immunology, University of Göteborg, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden

Received 3 January 1992/Accepted 14 April 1992

Vaccine antigen-specific antibody-secreting cell (ASC) responses in peripheral blood of healthy adult volunteers were studied after oral immunization with a prototype enterotoxigenic Escherichia coli (ETEC) vaccine by means of the enzyme-linked immunospot technique. Three doses of vaccine consisting of formalin-killed ETEC bacteria expressing fimbrial colonization factor antigens I and II (CFA/I and CFA/II) in combination with purified cholera toxin B subunit (CTB) were given 2 weeks apart. The ASC responses were detected 7 days after each immunization. Immunoglobulin A (IgA) was the predominant isotype produced by CFA/I- as well as CFA/II-specific ASCs. Moderate CFA/I- and CFA/II-specific IgM-secreting ASC (IgM-ASC) responses were also seen, whereas IgG-ASC responses to either of the CFAs were negligible. The ASC responses to CTB, on the other hand, comprised both IgA- and IgG-ASCs, with few if any specific IgM-ASCs. Almost 90% of the volunteers developed CFA-specific ASC responses after vaccination. Maximal CFA-specific ASC responses were usually observed after a single dose or two doses of vaccine. A third dose of vaccine did not result in increased but rather resulted in decreased magnitudes of CFA-specific ASC responses. Furthermore, it was found that CTB did not function as a mucosal adjuvant, since CFA-specific ASC responses were not enhanced by the simultaneous administration of CTB. These results suggest that two oral doses of ETEC vaccine induce a strong mucosal immune response, as reflected by the presence of large numbers of antigen-specific mucosal B cell immunoblasts in the blood.

It has been estimated that enterotoxigenic Escherichia coli (ETEC) give rise to 650 million cases of diarrhea and 800,000 deaths annually in children under 5 years of age in developing countries (2). In addition, ETEC are the predominant cause of diarrhea in travelers to these regions (3, 43). ETEC are noninvasive organisms that colonize the small intestine by means of fimbrial colonization factor antigens (CFAs) (16). Three major CFAs have been identified in 50 to 70% of human ETEC isolates: CFA/I, which is a homogeneous antigen; CFA/II, which consists of three subcomponents called colI surface antigens CS1, CS2, and CS3; and CFA/IV, which is composed of CS4, CS5, and CS6. Studies in humans and animals have shown that these CFAs give rise to immune responses which protect against subsequent challenge with ETEC expressing the corresponding fimbriae (15, 26, 40).

ETEC cause disease by elaborating one or two enterotoxins: a heat-labile toxin (LT) and a heat-stable toxin (ST). LT is structurally and immunologically closely related to cholera toxin (CT), and it has been shown that the B subunit of CT (CTB) provides protective immunity against E. coli LT disease (6, 33).

No human vaccine against ETEC disease is yet available. An ideal ETEC vaccine should probably consist of the major CFAs in combination with a toxoid in order to give rise to antitoxic as well as anticolonization immunity (37, 38). Furthermore, it should be given by the oral route in order to induce optimal mucosal immune responses in the gut.

Several studies have shown that oral immunization with microbial antigens can induce the appearance of specific antibodies in mucosal secretions (4, 31, 39) and antibody-secreting cells (ASCs) both in the circulation (11, 22, 45) and in the intestine (35). In this study, we demonstrate that oral immunization with a prototype ETEC vaccine containing formalin-killed CFA/I- and CFA/II-expressing bacteria in combination with CTB gives rise to vaccine-specific ASC responses against CFAs as well as CTB in peripheral blood of human volunteers.

MATERIALS AND METHODS

Subjects and immunizations. Thirty-seven healthy adult individuals, 22 women and 15 men, 20 to 55 years old, volunteered to participate in this study (which was approved by the Research Ethical Committee of the Medical Faculty, University of Göteborg). All but seven of them were of Swedish origin. Subjects with gastrointestinal problems or those who had travelled during the 3 months preceding the study to areas in which ETEC are endemic were excluded. Thirty-one volunteers received three oral doses of a prototype ETEC vaccine (37), with a 2-week interval between doses; six volunteers were studied for control purposes only. Each dose of vaccine consisted of 10^11 formalin-killed ETEC bacteria expressing CFA/I and CFA/II (CS1, CS2, and CS3) (1, 37). The following strains were used: an ST-positive O78:H12 strain expressing CFA/I (19), a toxin-negative O139:H28 strain expressing CS1, and an ST-positive O6:H16 strain expressing CS2 and CS3 (the latter two strains were kindly provided by B. Rowe and M. M. McConnell, Division of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom). The strains were grown under

* Corresponding author.
conditions leading to a high level of expression of the different CFAs, and thereafter the organisms were killed by mild formalin treatment, preserving 50 to 100% of the CFA activity as determined by a quantitative enzyme-linked immunosorbent assay using monoclonal antibodies to the different CFA/CS proteins (27, 37). The inactivated bacteria were mixed to give a total of 10¹¹ formalin-killed E. coli bacteria in 4 ml of phosphate-buffered saline (PBS), corresponding to one vaccine dose. To provide the CFA component, 21 of the vaccinees also received a dose of oral cholera vaccine, consisting of 1 mg of purified CTB and 10¹¹ killed *Vibrio cholerae O1* organisms (5) together with the ETEC expressing the various CFAs (CFA+ ETEC). The different vaccine components were mixed with 150 ml of a sodium bicarbonate-citric acid buffer (ACO Pharmachemicals, Stockholm, Sweden) and given as a drink; the volunteers were not allowed to eat for 2 h before and 1 h after vaccine administration. The CFA+ ETEC component was produced by the Swedish National Bacteriological Laboratory (Stockholm, Sweden), and the cholera vaccine was produced by Institut Mérieux (Lyon, France).

Samples of 20 to 30 ml of heparinized venous blood were collected 7 days after oral immunization from the volunteers for each enzyme-linked immunospot (ELISPOT) assay. The group of 21 vaccinees receiving both the CFA+ ETEC and the CFA component was bled as follows: 19 after the third immunization, 12 after the second immunization, and 7 after the first immunization. Finally, the group of 10 volunteers that only received the CFA+ ETEC component was bled after the second immunization.

**Surveillance of side effects.** Vaccines were monitored for the possible occurrence of fever, abdominal cramps, vomiting, loose stools, and hypersensitivity reactions during 7 days following each of the three immunizations.

**Purified antigens.** Purified CFA/I was prepared from a flagellum-deficient mutant of strain H10407 (O78:K80: H11,ST/LT) by homogenization with a Waring blender followed by ammonium sulfate fractionation and negative diethylaminoethyl-Sephadex column chromatography (14). Purified CFA/II (CS1 plus CS3) was prepared from strain E1392-75 (O6:K15:H16,ST/LT) by homogenization followed by salt precipitation and column chromatography (24). GM1 ganglioside was purchased from Sigma Chemical Co., St. Louis, Mo., and purified CT was purchased from List Biological Laboratories, Inc., Campbell, Calif.

**Detection of ASCs.** Peripheral blood mononuclear cells (MNC) were isolated from heparinized venous blood by gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) and were assayed for numbers of total and specific ASCs by a two-color micromodification (7) of the original ELISPOT assay (8). Individual wells of nitrocellulose-bottom 96-well plates (Millititer HA; Millipore Corp., Bedford, Mass.) were coated with 0.1 ml of purified CFA/I (10 µg/ml), CFA/II (20 µg/ml), or GM1 ganglioside (3 nmol/ml), diluted in PBS, overnight at 4°C. After PBS washes, the wells coated with GM1 were further exposed to CT (2.5 µg/ml) for 2 h at 37°C. Following three additional washes with PBS for 2 h at 37°C, the MNC suspensions was added to all wells, containing various numbers of cells (5 × 10⁴ to 1 × 10⁶), and the plates were incubated for 3 h at 37°C in a humidified atmosphere supplemented with 7.5% CO₂. Thereafter, a mixture of two affinity-purified goat anti-human immunoglobulin antibodies with distinct isotype specificities, one conjugated to alkaline phosphatase and the other conjugated to horseradish peroxidase, was added to the wells. The antibodies had specificity for the Fc fragment of human immunoglobulin A (IgA), IgM, or IgG and were purchased from Southern Biotechnology Associates, Birmingham, Ala. Plates were incubated overnight with the enzyme-conjugated anti-immunoglobulins at 4°C and were thereafter developed by the sequential addition of the appropriate enzyme chromogen substrates. Spots, corresponding to the zones of antibodies secreted by individual cells, were enumerated in quadruplicate wells under low magnification (×40), and data were adjusted to numbers of spot-forming cells per 10⁶ MNC.

Total IgA-, IgG-, and IgM-secreting cells were similarly enumerated in wells previously coated with affinity-purified goat antibodies to the F(ab')₂ fragment of human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). Exposure of cell suspensions to 2 mM cycloheximide (Sigma) 2 h prior to and during plating abolished spot formation by the MNC, demonstrating that only metabolically active ASCs were detected in this assay.

**Statistics.** Statistical differences between ASC responses in immunized and nonimmunized volunteers were determined by Wilcoxon's rank sum test. The statistical significance of the difference in ASC responses in 10 volunteers after two versus after three immunizations was determined by the Wilcoxon signed rank test.

**RESULTS**

The vaccine was well accepted: 2 of 31 vaccinees experienced slight abdominal discomfort for a couple of hours on the day of either the first or second immunization. In a parallel study, such symptoms have been observed after administration of the bifaricate component alone (36a). None of the vaccinees suffered from diarrhea or any other local or systemic adverse reactions.

**Circulating ASC responses to repeated immunizations.** In an initial study, we examined whether oral administration of three doses of a newly developed prototype ETEC vaccine would give rise to ASCs in the circulation of 19 adult Swedish volunteers. As shown in Fig. 1, the vaccine induced specific ASCs both against fimbrial antigens CFA/I and CFA/II and against CTB in the peripheral blood of most volunteers. CFA-specific ASC numbers were low to negligible in nonimmunized volunteers.

ASC responses to both CFAs were comparable in magnitude and isotype distribution, with IgA-ASCs dominating the response. After three oral immunizations, the vaccinees had, respectively, 14- and 11-times-higher geometric mean levels of IgA-ASCs directed against CFA/I and CFA/II than did nonimmunized controls. The geometric means of specific IgA-ASCs postvaccination were 31 per 10⁷ MNC for CFA/I and 23 per 10⁷ MNC for CFA/II (Fig. 1). Although less pronounced, IgM-ASC responses to the CFAs were also detected in most vaccinees (geometric means of 18 per 10⁷ MNC for CFA/I and 9 per 10⁷ MNC for CFA/II). After three immunizations, the geometric means of IgM-ASCs were 11 and 6 times higher for CFA/I and CFA/II, respectively, than in nonimmunized controls. CFA-specific IgG-ASCs were rarely detected (Fig. 1).

Specific ASC responses to the CTB component of the vaccine were detected in all volunteers but differed from CFA-specific responses with respect to isotype distribution. Thus, in keeping with the results of our previous study (11), CTB-specific responses comprised both IgA- and IgG-ASCs and few IgM-ASCs (Fig. 1).

Among the 21 volunteers examined after oral administration of the ETEC vaccine (i.e., CFA+ ETEC and CTB),
FIG. 1. Frequency of vaccine-specific IgA-, IgM-, and IgG-secreting cells in human peripheral blood 1 week after the third immunization with oral ETEC vaccine. Values are based on quadruplicate determinations for immunized (closed symbols) and non-immunized (open symbols) individuals. Asterisks denote a statistically significant (P < 0.001) difference between the geometric mean (vertical boxes) of ASCs of immunized versus nonimmunized individuals; n.s., not significant.

almost 90% responded with significantly increased frequencies of circulating IgA- and IgM-ASCs to CFA/I and CFA/II. A vaccine-specific ASC response exceeding by at least 2 standard deviations the geometric mean of ASCs of nonimmunized controls was considered a significant response. All volunteers were found to respond with circulating CTB-specific IgA- and IgG-ASCs (Table 1). Less than half of the vaccinees responded with IgM-ASCs to CTB, and these responses were rather low (Table 1 and Fig. 2).

We also evaluated whether individual vaccinees responded equally well to all antigens tested, i.e., whether a volunteer responding to one of the three antigens also responded well to the other two. About half of the volunteers responded equally well to CFA/I, CFA/II, and CTB, whereas no such pattern was seen among the remaining vaccinees.

Comparison of ASC responses after one, two, and three immunizations. In six vaccinees, anti-CFA/I, -CFA/II, and -CTB ASC responses in peripheral blood were monitored immediately before and 6 to 7 days after each of three oral immunizations with the ETEC vaccine. The frequency of total IgA-secreting cells was not increased after any of the immunizations, whereas a slight increase in total IgM-secreting cells was seen after the first two immunizations but not after the third dose (data not shown). In most instances, no vaccine-specific ASCs could be detected in peripheral blood prior to immunization. CFA-specific IgA-ASCs were already manifest 1 week after the first vaccine dose in all six volunteers, being at least fourfold higher than corresponding preimmune values (Fig. 2). In three of six volunteers, maximal IgA- and IgM-ASC responses to CFA/I and CFA/II were recorded after the first immunization and were not increased by second and third immunizations (Fig. 2). The magnitude of CFA-specific ASC responses of the IgA isotype in these volunteers was noteworthy. Thus, after a single immunization, as many as 2,000 CFA/I-specific ASCs per 10^7 MNC were noted in two of the volunteers, and 1,000 CFA/I-specific ASC per 10^7 MNC were seen in one (Fig. 2). For the three remaining vaccinees, maximal CFA-specific IgA-ASC as well as IgM-ASC responses were reached after the second or third immunization.

For the group of six vaccinees, CFA-specific ASC responses seemed to be somewhat lower after the third dose of vaccine than after the second (Fig. 2). We therefore compared the levels of CFA-specific ASCs after two and three immunizations for a larger group of volunteers, yet could not prove that the apparent decrease in magnitude of specific ASCs after the third dose of vaccine was statistically significant (Table 2).

We also monitored circulating ASC responses to the CTB

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Frequency (%)</th>
<th>IgA</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA/I</td>
<td>86 (18/21)</td>
<td>86 (18/21)</td>
<td>8 (1/13)^a</td>
<td></td>
</tr>
<tr>
<td>CFA/II</td>
<td>90 (19/21)</td>
<td>90 (19/21)</td>
<td>28 (4/14)^c</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>100 (21/21)</td>
<td>47 (9/19)^d</td>
<td>100 (15/15)</td>
<td></td>
</tr>
</tbody>
</table>

^a ASCs were monitored after either of the three oral vaccine doses. A vaccinee was considered to be a responder when displaying a frequency of vaccine-specific ASCs exceeding by at least 2 standard deviations the geometric mean of ASCs of a group of preimmune and/or nonimmunized controls.

^c Only specimens from 14 of the 15 vaccinees were analyzed because of limited cell yields.

^d Only specimens from 19 of the 21 vaccinees were analyzed because of limited cell yields.

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**TABLE 1. Frequency of vaccinees with significant ASC responses to CFA and CTB after immunization with the oral ETEC vaccine**

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component of the vaccine. The latter responses differed from the CFA-specific ASC responses with regard to both magnitude and isotype distribution. Thus, CTB-specific responses comprised IgA- and IgG-ASCs with few IgM-ASCs, and maximal responses were recorded after two or three doses of vaccine. For these six vaccinees, the maximal geometric mean increases of vaccine-specific ASCs were 35-fold for CFA/I (range of fold increase, 10 to 865), 117-fold for CFA/II (range of fold increase, 15 to 2,160), and 342-fold for CT (range of fold increase, 63 to 1,115).

Influence of the CTB component on CFA-specific ASC responses. To determine whether the CFA-specific ASC responses were enhanced by the CTB component of the vaccine, i.e., whether CTB might have acted as a mucosal adjuvant, we compared 12 vaccinees receiving both CFA+ ETEC and CTB, with 10 vaccinees receiving the CFA+ ETEC component alone. As shown in Table 3, there was no increase in the magnitude of CFA-specific ASC responses upon coadministration of CTB.

DISCUSSION

Extensive efforts have been devoted to the development of an ETEC vaccine that would stimulate a protective immune response in the human intestine. Several oral ETEC

![Graphs showing frequency of vaccine-specific IgA- and IgM-ASCs in peripheral blood of six volunteers, before and 1 week after each of three consecutive doses of oral ETEC vaccine (given 2 weeks apart). Values are based on quadruplicate determinations. The dotted area denotes the range of geometric mean +2 standard deviations of ASC numbers determined for nonimmunized individuals.](image)

**TABLE 2.** Specific IgA-ASC responses to CFAs in peripheral blood of 10 matched vaccinees after two and three immunizations with the oral ETEC vaccine

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Geometric mean of no. of IgA-ASC/10^6 MNC</th>
<th>Range</th>
<th>Geometric mean of no. of IgM-ASC/10^6 MNC</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA/I</td>
<td>47</td>
<td>28–80</td>
<td>26</td>
<td>19–34</td>
</tr>
<tr>
<td>CFA/II</td>
<td>64</td>
<td>51–80</td>
<td>20</td>
<td>12–34</td>
</tr>
</tbody>
</table>

* Differences between the second and third immunizations were not significant by the Wilcoxon signed rank test.

* Geometric mean ± 1 standard error of the mean.
vaccine formulations have been proposed (25, 37, 38). Thus, oral immunization with purified fimbriae has been attempted but with little success (15), probably because the fimbriae are rapidly destroyed by the proteolytic enzymes of the gastric milieu (25, 36). Live attenuated vaccines have also been constructed, but unacceptable side effects in the form of diarrhea have been reported among the vaccine recipients (25).

The present ETEC vaccine formulation offers the following advantages: the CFAs are not destroyed when incubated in gastric juice (38), the vaccinees have not suffered from any noteworthy side effects, and significant antibody responses against both CFAs and CTB developed in the intestinal fluid of most recipients of this vaccine (1).

According to the concept of a common mucosal immune system, antigen-sensitized B cells from the intestine will transit through the circulation and thereafter home to the gut and other mucosal tissues of the body for terminal differentiation into fully immunocompetent B cells (21, 29). This concept has led to a growing interest in the possibility of detecting vaccine-induced ASCs in the circulation for the evaluation of the immunogenicity of mucosal vaccines in humans (9-11, 17, 22, 45, 48). The aim of this study was to examine the extent to which this prototype ETEC vaccine could evoke a mucosal immune response, monitored by the presence of gut-derived ASCs in the blood. Oral administration of the vaccine did indeed give rise to ASCs in the blood with specificity for the most important vaccine components, i.e., CFA/I, CFA/II, and CTB. The CFA-specific ASC response was characterized by a dominance of IgA-ASCs, followed by IgM-ASCs; IgG-ASCs were rarely detected. This isotype distribution is typical of a mucosal immune response and differs from the ASC response to the CTB component, which comprised both IgA- and IgG-ASCs and very few IgM-ASCs. There may be several explanations for this difference. First, uptake and processing of CFA+ ETEC and soluble CTB in the intestine may involve different mechanisms, resulting in distinct pathways of antigen presentation. Eldridge et al. (12) have shown in a murine system using biodegradable microspheres that only particles less than 5 μm in diameter were taken up by the Peyers’ patches and transported within macrophages to the mesenteric lymph nodes and spleen. The superior capacity of CTB compared with CFA+ ETEC in inducing a systemic immune response may be ascribed to differences in size. The CTB molecule is 8 nm in diameter (32), whereas an ETEC bacterium expressing CFA measures about 2 μm by 5 μm (44). Second, uptake of CTB is believed to involve binding to cell surface GM1 ganglioside receptor, which is expressed on both epithelial enterocytes and certain lymphoid cells, including antigen-presenting cells (macrophages and B cells) (18, 20, 30). The large numbers of circulating CTB-specific IgG-ASCs detected in this study may in fact indicate that a substantial proportion of these cells have been initially recruited and further expanded in mesenteric lymph nodes. Recent investigations in subhuman primates indicate that mesenteric lymph nodes of animals orally immunized with CT are the major site of CTB-specific IgG-ASCs (11a). Furthermore, CFAs are exposed on the bacterial cell surface together with lipopolysaccharide, which might have acted as a nonspecific polyclonal activator, thereby influencing the size and isotype distribution of vaccine-specific immune responses. However, this is unlikely in view of the finding that the frequencies of total IgA-secreting cells were not influenced by the immunization regimen. In addition, CTB has been shown to have immunomodulatory properties: it has been demonstrated in murine systems that CTB influences isotype switching (28) and may act as an adjuvant to mucosally administered antigens (13, 41, 42). The possibility that CTB might have acted as a mucosal adjuvant is, however, unlikely, since the CFA-specific responses were comparable in magnitude among vaccinees receiving the CFA+ ETEC component alone and those receiving it together with CTB.

The results of this study suggest that two oral immunizations are efficient at inducing optimal CFA-specific responses, since the numbers of CFA-ASCs were not increased but rather were decreased upon administration of a third dose of vaccine. Similar results have been obtained in analyses of antibody responses in intestinal lavage fluid after two versus three immunizations with this vaccine (1). Previous reports have indicated that individuals with high levels of preexisting antibodies to vaccine components might become refractory to oral immunization with the same antigens (9, 46). This decreased ability to respond to orally administered antigens may be due to secreted intestinal IgA (and IgM) antibodies which could interfere with the absorption of antigen in the intestine (23, 34, 47). The fact that the CTB-specific ASC responses were enlarged after both a second and a third dose of vaccine may be explained by receptor availability: GM1 ganglioside is expressed at high densities on epithelial enterocytes (20). Thus, despite the presence of secreted CTB-specific antibodies in the intestinal lumen, CTB might still be able to be absorbed by the intestine (thereby further boosting CTB-specific mucosal immune responses).

The magnitudes of the circulating ASC responses induced by the ETEC vaccine were comparable to or slightly lower than the peripheral blood ASC responses seen after oral immunization with other killed or live attenuated bacterial vaccines (9, 22, 45). However, the CFA-specific ASC responses observed in this study have probably been underestimated, since they were often monitored after the third immunization and maximal CFA-specific responses seem to occur after the first or second immunization. Furthermore, we determined only responses to purified antigens, whereas in most other studies, ASC responses have been recorded to whole-cell bacterial antigens. Finally, responses were considerably higher in pre- and postimmunization samples from the same person than in samples from vaccinated and nonvaccinated groups.

This study lends further support to the assumption that determinations of specific B-cell responses in peripheral blood, the most readily available lymphoid compartment in humans, are reliable proxy measures of the immunogenicity of mucosal vaccines. This prototype ETEC vaccine elicited circulating ASC responses to the most important protective
antigens and with an isotype distribution typical of a mucosal immune response. Further support for the assumption that the circulating B-cell precursors that we monitored were indeed derived from the intestine is that volunteers who responded with vaccine-specific ASCs also had vaccine-specific IgA in intestinal fluid and usually responded poorly in serum (1).

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