Antibody-Producing Cells in Peripheral Blood and Salivary Glands after Oral Cholera Vaccination of Humans

CECIL CZERKINSKY,1* ANN-MARI SVENNERHOLM,1 MARIANNE QUIDING,1 ROLAND JONSSON,2 AND JAN HOLMGREN1

Department of Medical Microbiology and Immunology1 and Department of Oral Pathology,2 University of Göteborg, Guldhedsrgatan 10A, Göteborg, Sweden

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We examined whether immunization with a newly developed oral cholera vaccine would elicit gut-derived antibody-producing cells in the blood and in distant mucosal tissues, such as the minor salivary glands, in 30 adult Swedish volunteers. The results of this study demonstrated that this vaccine indeed induced production of specific antibody-producing cells against the cholera toxin B subunit in both peripheral blood and salivary glands. The response in blood, which after primary and booster immunizations comprised both immunoglobulin A (IgA) and IgG antibody-forming cells, was highly transient and preceded the response in salivary glands; the latter response was restricted to the IgA isotype. The results provide further evidence of the existence of a common mucosal immune system in humans. Furthermore, these findings support previous observations that in animals, the cholera toxin B subunit may be a useful carrier protein for preparing enteric vaccines against pathogens encountered at intestinal and extraintestinal mucosal sites.

MATERIALS AND METHODS

Subjects and immunizations. Thirty healthy adult Swedish individuals, 18 females and 12 males, 26 to 42 years old, volunteered for this study (which was approved by the Research Ethical Committee of The Medical Faculty of the University of Göteborg). Twenty-three volunteers received two or three peroral immunizations with a recently developed cholera vaccine (14), each dose consisting of 1 mg of purified CT B subunit and 1 × 1011 killed cholera vibrios administered in 150 ml of alkalizing buffer (bicarbonate) (31). The time interval between each dose was 2 weeks. The vaccine was produced by Institut Mérieux (Lyon, France), and the B subunit was prepared according to previously published large-scale purification protocols (35). The vaccine used in this study was of the same lot that has been extensively tested in a large field trial in Bangladesh (31). Samples of unstimulated parotid saliva (9, 22) and venous blood were collected before immunization and at weekly intervals thereafter. Saliva samples were inactivated at 56°C for 60 min and clarified by centrifugation at 9,000 × g for 10 min. Serum and saliva samples were kept frozen (−20°C) until used for enzyme-linked immunosorbent assay (ELISA) titrations (see below).

Isolation of MNC. Peripheral blood mononuclear cells (MNC) were isolated by centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) (3), washed twice with 0.01 M phosphate buffer−0.15 M NaCl, pH 7.4 (PBS), and kept on ice until assayed.

Minor salivary glands were obtained from each of the volunteers on one occasion. From each volunteer, 6 to 10 glands were collected by superficial resection of the lower lip, under local anesthesia. The glands were extensively rinsed with ice-cold PBS containing 10 mM dithiothreitol to remove surrounding mucus and connective tissue debris. Glands were then sliced to a thickness of 150 by 100 μm by using a semiautomated tissue chopper (McIlwain, Gilford, United Kingdom). The fragments were resuspended with cold Ca2+- and Mg2+-free Hanks balanced salt solution (GIBCO Europe, Glasgow, United Kingdom) with 10 mM dithiothreitol and washed twice by centrifugation (250 × g, 5

* Corresponding author.
min, 4°C). The fragments were then placed in a siliconized flask and were resuspended with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (25 mM)-buffered Hanks balanced salt solution containing CaCl₂ (1 mM), dithiothreitol (10 mM), and *Bacillus thermoproteolyticus* thermolysin (Boehringer Mannheim, West Germany) (0.5 mg/ml), pH 7.4. Following incubation for 30 min at 4°C under continuous shaking, single cells were recovered by passing the contents of the digestion vessels through a thin nylon net (50-μm mesh). The procedure was repeated once more on the remaining fragments. Immediately after each digestion cycle, cells were pelleted by centrifugation (400 × g, 5 min) through a cushion of fetal calf serum (FCS). Pelleted cells were resuspended and incubated for 20 min at 37°C with 1.5 ml of Iscove’s medium (GIBCO) containing 1 mg of deoxyribonuclease (type I; Sigma Chemicals, St. Louis, Mo.) per ml and 10% FCS. The cell suspensions were then washed by centrifugation (400 × g, 20 min, 10°C) through a cushion of isotonic, isoosmolar 30% Percoll (Pharmacia) solution. The pelleted cells were then resuspended with HEPES-buffered Iscove’s medium containing 10% FCS and antibiotics and kept on ice prior to being assayed (see below). On average, the above extraction procedure yielded 4.8 × 10⁶ ± 1.9 × 10⁶ viable (trypan blue excluding) nucleated cells per pool of 10 glands, of which approximately 20 to 30% were MNC, 60 to 70% were epithelial cells, and less than 5% were fibroblasts and undifferentiated cells.

**Detection of antibody-producing cells.** Peripheral blood and salivary gland cell suspensions were assayed for numbers of total and specific antibody-secreting cells by a two-color micromodification (7) of the original enzyme-linked immunosorbent assay (ELISA) (8, 28). Individual wells of nitrocellulose-coated 96-well Militter hemaggulination plates (Millipore, Bedford, Mass.) were coated with GM1 ganglioside (3 nM/ml, overnight at 4°C), washed 3 times with PBS, and further exposed for 3 h at ambient temperature to 0.1 ml of PBS containing 2.5 μg of purified CT (List Biological Laboratories, Inc., Campbell, Calif.) or 10 μg of bovine serum albumin (control wells). Unadsorbed proteins were removed by three successive washings with PBS, and the wells were filled with 0.2 ml of PBS containing 1% FCS and incubated at 37°C for 2 h. The contents of the wells were replaced with 0.1 ml of cell suspensions containing various numbers of MNC. Plates were then incubated undisturbed for 16 h at 37°C in a humidified atmosphere with 7% CO₂. Plates were then rinsed three times with PBS and four times with PBS containing 0.05% Tween 20. Next, 0.1 ml of PBS–0.05% Tween 20 containing 1% FCS and either a mixture of affinity-purified goat anti-human IgG (0.8 μg/ml) and goat anti-human IgA (1.6 μg/ml) antibodies conjugated with alkaline phosphatase and horseradish peroxidase, respectively, or a mixture of affinity-purified alkaline phosphatase-conjugated goat anti-human IgA (0.8 μg/ml) and horseradish peroxidase-conjugated anti-human IgM (1 μg/ml) antibodies were added to the wells. All enzyme-conjugated antoglobulin reagents were purchased from Southern Biotechnology Associates (Birmingham, Ala.), and their specificities were confirmed by ELISA block titrations against a panel of human IgA and IgM paraproteins (gifts from Jiri Mestecky, University of Alabama at Birmingham) and purified human polyclonal IgG. Plates were incubated with enzyme-conjugated antiglobulins for 2 to 3 h at ambient temperature and washed four times with PBS and twice with 0.05 M Tris-buffered saline, pH 8.0. Wells were then decanted and exposed to 0.1 ml of alkaline phosphatase chromogen substrate for 10 to 20 min, washed with PBS, and exposed to 0.1 ml of horseradish peroxidase chromogen substrate for 5 to 10 min (7). Dishes were thoroughly rinsed with tap water, dried, and examined for the presence of blue (alkaline phosphatase) and red (horseradish peroxidase) spots. These reactions were enumerated under low magnification (×40). Numbers of spot-forming cells (SFC) were determined on duplicate (for salivary gland suspensions) or quadruplicate (for peripheral blood suspensions) wells and adjusted to numbers of SFC 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 (5 μg/ml) (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.).

Exposure of cell suspensions to cycloheximide (2 × 10⁻³ M) for 3 h at 37°C before and during cell plating abrogated CT-specific as well as total immunoglobulin-mediated spot formation, thus demonstrating that this assay detects cells synthesizing and actively secreting immunoglobulins.

**ELISAs.** Serum and parotid saliva samples were assayed by ELISA for levels of IgA and IgG antibodies to CT attached to GM1 gangliosides, as previously described (32).

**RESULTS**

Circulating antibody responses to oral cholera vaccine. We examined whether single or repeated oral immunizations with the B subunit-containing cholera vaccine would elicit an antitoxin SFC response in peripheral blood and to what extent the magnitude, kinetics, and isotype distribution of this response corresponded to the antibody response in serum and to the responses in salivary glands and in parotid saliva (see below). The serological analyses showed that in all vaccinees, a significant serum antitoxin antibody response developed after the first immunization (Table 1). This response was slightly increased by a second and third immunization. Antibodies of the IgG isotype predominated in the serological responses of all individuals, showing a 6-fold geometric mean increase in titer from preimmunization levels after the first oral dose of cholera vaccine and showing 10- and 12-fold increases after the second and third immunizations, respectively. In the majority of volunteers, the oral cholera vaccination also induced an IgA antitoxin response in serum. Kinetically, the IgA response followed the IgG response but was of a lower magnitude and occurred less frequently than the IgG response (Table 1). The geometric mean increases in titer of serum IgA antitoxin antibody responses after the first, second, and third immunizations were 1.3-, 4.0-, and 10-fold, and the increases in frequency of responses were 15, 50, and 75%, respectively.

The SFC responses in peripheral blood differed sharply from the serological responses with regard to kinetics and isotype distribution (Fig. 1). While the serum antibody titers were not significantly increased until 2 weeks after the first immunization, SFC responses were already manifest 1 week after primary immunization and then decreased to almost background level after another week. This kinetically dramatic SFC response with a brisk but transient elevation in SFC was then repeated and slightly enlarged after each of the booster immunizations (Fig. 1). Also in contrast to the serological response, the frequencies of IgA and IgG SFC were comparable. In no instance could IgM SFC be detected in the peripheral blood of oral cholera vaccinees throughout the study.

Salivary antibody responses to cholera vaccine. ELISA
analyses of parotid saliva specimens confirmed previous observations that peroral immunization with the cholera vaccine can result in increased IgA as well as IgG antibody levels in saliva (33). Thus, although the specific salivary antibody titers did not increase significantly in the 2 weeks after the first immunization, there was a three- to fourfold geometric mean increase in titer in both IgG and IgA antitoxin levels after the second immunization and a fivefold rise after the third vaccination (Table 1). Salivary IgA antitoxin titers were consistently higher than the corresponding IgG titers. Determinations of SFC in minor salivary glands revealed that the increase in salivary antitoxin antibodies was associated with an active B-cell response in minor labial salivary glands (Table 2). However, the minor salivary gland-specific antitoxin SFC response was restricted to the IgA isotype (Table 2). Strong individual variations in the magnitudes of the IgA SFC responses were observed. Although no biopsies were taken between the first and second immunizations, a substantial IgA antitoxin SFC response was observed in three of four volunteers examined 7 days after the second immunization. Only one of three volunteers examined 1 week after the third immunization demonstrated increased numbers of IgA antitoxin SFC in labial salivary glands. Specific IgG SFC were undetectable in minor salivary glands (Table 2), albeit substantial numbers of total IgG-producing cells were seen in these glands (248 ± 105 IgG SFC per 10^6 MNC versus 872 ± 316 IgA SFC per 10^6 MNC; n = 22). This contrasted with the serological response in parotid saliva and in serum (Table 1), as well as with the B-cell response in peripheral blood (Fig. 1), in which both IgA and IgG antitoxin antibodies were detected. It was also evident that the SFC response in minor salivary glands peaked at a later time than in blood, especially 2 to 3 weeks after the third immunization.

### TABLE 1. Antibody responses in serum and in parotid saliva after oral immunizations with combined CT B subunit-whole-cell vaccine

<table>
<thead>
<tr>
<th>Day post-primary immunization (n)</th>
<th>Mean antibody titer ± SD (no. with significant increase in antibody titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum IgG</td>
</tr>
<tr>
<td>0 (8)</td>
<td>115 ± 50</td>
</tr>
<tr>
<td>7 (8)</td>
<td>110 ± 50 (1)</td>
</tr>
<tr>
<td>14 (6)</td>
<td>675 ± 275 (6)</td>
</tr>
<tr>
<td>21 (10)</td>
<td>1,175 ± 350 (10)</td>
</tr>
<tr>
<td>28 (10)</td>
<td>1,100 ± 470 (9)</td>
</tr>
<tr>
<td>35 (13)</td>
<td>700 ± 360 (10)</td>
</tr>
<tr>
<td>44 (12)</td>
<td>1,450 ± 480 (12)</td>
</tr>
<tr>
<td>56 (8)</td>
<td>580 ± 270 (7)</td>
</tr>
</tbody>
</table>

a Days 14 and 28 correspond to the second and third oral vaccinations, respectively.

b Antibody titers represent the reciprocal dilution of serum or saliva giving an ELISA absorbance value more than twice that of the control (no sample added).

A significant increase in antibody titer was defined as greater than twofold.

P < 0.05.

P < 0.01.

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**FIG. 1.** Frequency of CT B subunit-specific antibody-secreting cells in human peripheral blood after oral immunization with a combined CT B subunit-whole-cell vaccine. Results are expressed as mean number of SFC per 10^6 MNC, determined on quadruplicate wells for each individual. Numbers of SFC in control (bovine serum albumin-coated) wells were always lower than 5 SFC per 10^6 MNC and were subtracted from numbers of SFC in CT B subunit-coated wells. The horizontal dotted line denotes the upper limit (±5 SFC per 10^6 MNC), below which net values were considered negative (99% confidence interval). Symbols: •, IgA; ○, IgG; □, IgM.
TABLE 2. Frequency of CT B subunit-specific antibody-secreting cells in minor labial salivary glands after oral immunizations with combined CT B subunit-whole-cell vaccine

<table>
<thead>
<tr>
<th>Day post-primary immunization (n)</th>
<th>No. of CTB-specific SFC/10^6 MNC^a</th>
<th>IgA</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not immunized (7)</td>
<td></td>
<td>2 ± 6</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>21 (4)</td>
<td></td>
<td>35 ± 14^b</td>
<td>1 ± 4</td>
</tr>
<tr>
<td>28 (4)</td>
<td></td>
<td>1 ± 10</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>36 (4)</td>
<td></td>
<td>10 ± 8</td>
<td>1 ± 6</td>
</tr>
<tr>
<td>44 (4)</td>
<td></td>
<td>30 ± 8^b</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>56 (3)</td>
<td></td>
<td>10 ± 5</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>77 (4)</td>
<td></td>
<td>1 ± 4</td>
<td>1 ± 4</td>
</tr>
</tbody>
</table>

^a Data are expressed as mean no. of SFC ± standard deviation adjusted per 10^6 salivary gland MNC. Days 14 and 28 correspond to the second and third oral vaccinations. ND, Not determined.

^b Significant increase over non-immune values. *, P < 0.05 (Wilcoxon rank sum test).

immunization, at which time the blood SFC response had essentially disappeared. Furthermore, control salivary gland biopsies done before any intentional immunization were completely devoid of antitoxin SFC.

DISCUSSION

The finding that ingestion of antigens results in the selective induction of IgA antibodies in human external secretions (21), together with data from animal studies showing migration of Peyer’s patch B cells to various mucosal tissues (1), has suggested that antigen taken up by intestinal M cells sensitizes IgA-committed B-cell precursors which thereafter migrate to distant mucosal sites and generate local secretory IgA antibody responses (5). Evidence for a common mucosal immune system in humans has been scanty because of the difficulty in demonstrating migratory behavior of mucosal B cells. Recent studies have demonstrated the transient appearance of antigen-specific IgA antibody-secreting cells in peripheral blood after mucosal immunization of humans (9, 15, 17, 39). These observations provide support for the existence of a common mucosal immune system in humans, but the evidence remains indirect in the absence of demonstration of migration of gut-derived IgA precursors to nonintestinal mucosal or glandular tissues and of their differentiation there. The main finding of the present study is that following oral immunization of humans with the B subunit-containing cholera vaccine, there was a substantial IgA antitoxin-producing cell response in minor salivary glands preceded by a brisk and transient specific SFC response in blood. In contrast to the blood SFC responses which comprised both IgG SFC and IgA SFC and to the serological responses which either were dominated by IgG antibodies (in serum) or consisted of both IgG and IgA (in parotid saliva), the SFC response in minor salivary glands was confined to the IgA isotype. This suggests marked differences in the migratory properties of IgA- and IgG-committed B cells activated at gut sites.

The extent to which IgG and IgA antitoxin antibodies detected in parotid saliva are locally synthesized and/or transferred from the intravascular pool is unknown. The fact that intravascular polymeric IgA is not efficiently transported into human parotid saliva, whereas a small fraction of IgG can transude from plasma to saliva (16), suggests that the anti-CT activity detected in parotid saliva is contributed by locally produced secretory IgA and serum-derived IgG. This interpretation is supported by other studies (24, 36) and by our findings showing a virtual absence of CT-specific IgG SFC in labial salivary glands despite substantial numbers of total IgG-producing cells in these tissues. The observation that the frequency of significant increases in IgG antitoxin antibody titer differed between serum and parotid saliva may be explained by variations among individuals in the capacity of the parotid glands to transport serum-derived IgG into the oral cavity. Similarly, the fact that IgA anti-CT B subunit antibody levels in parotid saliva and IgA anti-CT B subunit SFC numbers in minor salivary glands peaked at different times suggests that differences may exist between various salivary glands with regard to kinetics and rates of IgA production. This interpretation does not exclude the possibility that a small yet detectable proportion of the IgA antibody activity detected in parotid fluid is transported as polymeric IgA from the intravascular compartment. The substantial numbers of minor salivary gland cells producing IgG detected in this study are in keeping with recent serological analyses (29) and immunohistological studies (18, 23) and may reflect local stimulation of minor salivary gland lymphoid tissue by environmental antigens present in the salivary ducts (25).

While the serological antibody titers after immunization did not fluctuate much once they had been elevated (except for a slight additional increase with boosting), the kinetics of the cellular response was much more dramatic. In peripheral blood, there was an SFC peak 1 week after each of the vaccinations followed by a rapid decline until a new dose of vaccine was given. The SFC response in minor salivary glands was also transient, though appearing later than that in peripheral blood. These results are consistent with the notion that with each new encounter of the CT B subunit antigen at the level of the gut mucosa, a new set of activated B cells is released into the circulation. A substantial proportion of these cells differentiates to produce antibodies of the IgA isotype. Most likely, the majority of the IgA SFC recruited into the peripheral circulation is subsequently deposited in, and possibly further expanded in, mucosal and glandular tissues belonging to the mucosa-associated lymphoid tissues. They primarily provide a secretory antibody response in the gut mucosa (26a), but as indicated by the present data, they are also deposited in significant numbers in nonintestinal mucosal tissues including the salivary glands. Our results thereby lend direct support to the concept of a common mucosal immune network in humans. We regard the alternative interpretation that the immunization directly stimulated minor salivary gland lymphocytes (25) as unlikely, in view of both the minute fraction of the vaccine that could have reached the labial glands and the kinetics of the salivary gland SFC response, which was slower than that in both the gut and the blood (10). The possibility that the tonsils may provide a site of antigen uptake and may constitute an additional precursor source for a fraction of the B cells homing to the salivary glands (4) cannot be ruled out at this point.

Our findings of a substantial specific IgA SFC response in salivary glands after peroral immunization with cholera vaccine also lend support to the notion that enteric immunization with pertinent antigens coupled (chemically or by gene fusion) to the CT B subunit may be useful for stimulating an immune response against microbial pathogens encountered in the oral cavity. In mice, oral immunization with a protein antigen from Streptococcus mutans coa-
lently coupled to the CT B subunit evoked a substantial IgA SFC immune response in salivary glands against both the B subunit and the streptococcal protein antigen (11). On the basis of the present data, it seems reasonable that a similar response might also be achieved in humans with such conjugate vaccines. Our results therefore provide a basis for further efforts to prepare such conjugate vaccines for clinical trials in humans.

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REFERENCES


